Antiviral Research 99 (2013) 12–17

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Implications of baseline polymorphisms for potential resistance to NS3 protease inhibitors in Hepatitis C virus genotypes 1a, 2b and 3a

Navaneethan Palanisamy [a], Axel Danielsson [a], Chakradhar Kokkula [a], Hong Yin [a], Kåre Bondeson [a], Lars Wesslén [b], Ann-Sofi Duberg [c], Johan Lennerstrand [a,*]

[a] Clinical Virology, Department of Medical Sciences, Uppsala University, SE-751 85 Uppsala, Sweden
[b] Department of Infectious Diseases, Gävle Hospital, SE-801 87 Gävle, Sweden
[c] Department of Infectious Diseases, Örebro University Hospital, SE-701 85 Örebro, Sweden

ARTICLE INFO

Article history:
Received 25 January 2013
Revised 12 April 2013
Accepted 23 April 2013
Available online 3 May 2013

Keywords:
Hepatitis C virus
NS3 protease inhibitors
Resistance
Resistance associated amino acid variants (RAVs)
Prevalence

ABSTRACT

The future interferon-free treatment of hepatitis C virus (HCV) infection could include NS3 protease inhibitors (PIs) for potent pan-genotypic effect. We studied the prevalence of pre-existing PI resistance associated amino acid variants (RAVs) in 126 treatment-naive patient samples of HCV genotypes 1a, 2b and 3a, the most common genotypes in Sweden. The NS3 genes were each amplified by nested PCR method with degenerated primers to enable a broad genotype analysis. Population sequencing method was used, and the sequences were aligned with the NS3 sequence from HCV genotype 1a H77 strain. Interpretation of fold-change resistance to NS3 candidate drugs were done from already published phenotypic resistance data. The prevalence of known PI RAVs at baseline in genotype 1a was 28% (15/53), either single (V36L or Q80K/R) or combinations (T54A/S and V55A/I) of mutation(s). In genotype 2b, specific mutations like V36L, Q80G and S122R of viral NS3 protease gene were found in 100% (11/11). These may be the natural polymorphisms unique to genotype 2b. Similarly, specific mutations like V36L and D168Q were found uniquely in all 3a samples (30/30). The natural PI RAVs found in genotype 1a, although with relatively weak resistance, could still render up to 10-fold-resistance to the approved (boceprevir and telaprevir) and the 2nd generation PIs (faldaprevir and simeprevir). Moreover, the natural polymorphisms in genotype 2b (i.e. S122R) and 3a (i.e. D168Q), with inherent PI drug resistance of up to 20 and 700 fold respectively, would explain why current PIs are primarily directed against genotype 1.

1. Introduction

Hepatitis C virus (HCV) belongs to the *Flaviviridae* family, with its genome consisting of a positive-sense, single-stranded RNA. Globally, 170 million people are infected, with approximately 120–140 million chronic HCV carriers (Te and Jensen, 2010). In Sweden, the prevalence is estimated to be about 0.5%, which is approximately 45,000 HCV infected individuals (Duberg et al., 2008; Norda et al., 1995; Shev et al., 1995). The Uppsala–Gävle–Örebro region has a population of 1.4 million people, which is equivalent to approximately 7000 HCV infected individuals. The disease spreads through blood and blood products (Gravitz, 2011; Shepard et al., 2005). The developments in HCV diagnostic assays and the stringent rules in blood donation, have reduced the transmission of HCV via blood transfusions in Western countries (Prati, 2006). In Sweden, HCV infection is predominantly found in drug abusers due to the practice of sharing needles or paraphernalia (Månsson et al., 2000).

Treatment for HCV is complicated by the existence of several genotypes. HCV is classified into 7 genotypes (1–7) and >100 subtypes, and genotype 1 (a and b) is the most common in the Western countries (Nakano et al., 2012). The standard of care treatment (SOC) is based on pegylated-interferon-α and ribavirin. This combination therapy cures almost 80% of patients with HCV genotype 2 or 3, however, more than 50% of the genotype 1 infections are unresponsive to this treatment regimen (Manns et al., 2001). The reasons for treatment failure are mainly attributed to the inefficiency against genotype 1 (and genotype 4), but also, to some extent to the side effects (Hadziyannis et al., 2004; Kowdley, 2005). Interferon acts through the host immune system by enhancing the host immunity. The mode of action of ribavirin is still speculated, but it is not directly targeted to the HCV virus. Pharmaceutical companies are developing drugs that directly target specific HCV proteins like NS3 protease, NS5B polymerase and NS5A, all of them essential for HCV replication. In the summer of 2011, the first two HCV NS3 protease inhibitors (PIs), namely boceprevir and telaprevir, were approved in combination with

* Corresponding author. Address: Department of Medical Sciences, Section of Clinical Virology, Uppsala University, SE-751 85 Uppsala, Sweden. Tel: +46 186110265.
E-mail address: johan.lennerstrand@medsci.uu.se (J. Lennerstrand).

0166-3542/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.04.018

pegylated-interferon-α and ribavirin for treatment of chronic HCV genotype 1 infection (Bacon et al., 2011). A new generation of directly acting antivirals (DAAs) will be available in the clinics by 2013–2015. For example, the PIs simeprevir, faldaprevir (previously BI-201335), and vaniprevir, are currently in phase 3 trials. PIs are genotype specific to mainly type 1, whereas NS5B polymerase inhibitors (i.e. nucleos(t)ide analogues) and NS5A replication complex inhibitors have a broader range of activity (genotype 1–6) (Soriano et al., 2011). However, studies have demonstrated that simeprevir (PI) is fairly active against most genotypes with exception for 3a (Lenz et al., 2010), and lately, in a phase 2 trial, the novel protease inhibitor MK-5172 showed an even broader activity across genotypes than simeprevir (Summa et al., 2012).

In HCV infection, a high rate of viral turnover coupled with the error-prone viral NS5B RNA polymerase will result in a rapid accumulation of mutations (Martell et al., 1992). A study showed that in comparison to HIV and HBV, HCV has the most error-prone polymerase with the highest ability to develop resistance to DAAs when given as monotherapy (Soriano et al., 2008). With the use of *in vitro* assays (replicon), a variety of mutations associated with reduced susceptibility to DAAs have been identified in clinical plasma samples (Lagace et al., 2012; Lenz et al., 2010; Lin et al., 2004). Thus, the 1st generation PIs, boceprevir and telaprevir, are only approved to be used in combination with SOC; pegylated-interferon-α and ribavirin, to minimize the development of resistance. Later on, interferon free treatment will be available with a combination of 2nd generation DAAs, e.g. NS3 PI, NS5A, NS5B nucleoside and non-nucleoside inhibitors (Poordad and Chee, 2012; Hagan and Schinazi, 2013).

Due to the high sequence diversity of HCV, naturally occurring pre-existing resistance mutations have been found at low prevalence in HCV from treatment-naive patients (Bartels et al., 2008, 2013; Kuntzen et al., 2008). This was more prevalent for PIs than for NS5B polymerase nucleoside analogue inhibitors, because PIs involve less conserved binding sites than nucleoside inhibitors (Soriano et al., 2011). In one study, 9% of the PI untreated patients with genotype 1a infection had at least one pre-existing PI RAV in dominant form (Kuntzen et al., 2008). They used the normal population DNA sequencing method with capillary electrophoresis similar to the one used in our study, i.e. with a 20–25% detection limit of mixes. Lately, methods using deep sequencing, with a detection limit of mixes as low as of <0.1%, have showed the possibility to detect most RAVs (including the high resistance variants i.e. at position 155, 156 and 168) in every PI untreated genotype 1 patient (Thomas et al., 2012).

Our aim was to study the prevalence of naturally occurring mutations that could confer PI resistance in HCV infected patients with genotype 1a, 1b, 2b and 3a, in the Uppsala–Gävle–Örebro region of Sweden. We interpreted the fold resistance data of PIs for these HCV infected treatment naive patients, from already published phenotypic resistance data with genotype 1.

2. Materials and methods

2.1. Patients and sample collection

This study was ethically approved by the Regional Research Ethics Committee in Uppsala, Dnr 2009/023. The blood samples from HCV infected patients were collected during 2005–2011, and the serum was stored at −20 °C. The patients were all treatment naive to PIs, whereas only a few had received interferon and ribavirin regimen.

The serum blood samples were collected from 126 PI treatment-naive HCV infected patients at the city hospitals in Uppsala, Gävle and Örebro. The aim was to have at least 100 samples sequenced. We adopted the RT-nested PCR method, followed by sequencing (population sequencing) the whole HCV NS3 protease gene and performing the interpretation of resistance mutations with literature sources. Of the 126 patient samples, we could obtain sequences with good quality for 99 patient samples, where good quality was defined by the SeqScape® software as >20% tolerance for improper sequencing. The reason for not being able to study more than 99 sequences might be the result of the degenerated primers (Table 1) not being able to detect all the target regions, and also the use of random hexamers during the cDNA synthesis.

Table 1
Primer sequences for HCV NS3 gene used during 1st and 2nd round of nested-PCR.

Primer	Sequence 5′ to 3′	Position in HCV genome
1st Forward	ATCACsTGGGGrGCrGAyAC	3238–3258 (in NS2 region)
1st Reverse	AAyTTGCCrTAkGTGGAGTAyGT	4162–4185 (in NS3 region)
2nd Forward	ACsGCrGCrTGygGGGACAT	3257–3276 (in NS2 region)
2nd Reverse	GTGCTCTTrCCGCTrCCrGT	3983–4004 (in NS3 region)

2.2. RNA extraction, reverse transcription (RT) nested PCR and sequencing

RNA extraction from the serum samples (500 μL) was done using BioMérieux's NucliSens® easyMAG™ system as per the manufacturer's guidelines. The extracted RNA (20 μL) was stored at −70 °C. We synthesized cDNA from RNA template (9 μL) with SuperScript™ III One-Step PCR System (Invitrogen) using random hexamers. RT (reverse transcription) conditions consisted of pre-incubation at 25 °C for 10 min, followed by incubation at 42 °C for 60 min, final incubation at 85 °C for 5 min (to stop the reaction), and later at 4 °C for storing. Nested-PCR was performed with in-house primers targeting parts of the NS3 region using the TaqMan® Universal PCR Master Mix (Applied Biosystems). Five microliter of cDNA was used for the 1st round of nested-PCR. Thermocycling conditions for the 1st round of nested-PCR consisted of 1 cycle at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s; 50 °C for 30 s; 72 °C for 1 min; a final extension cycle (72 °C for 5 min), and a hold at 4 °C. Two microliter of 1st round PCR product was used for the 2nd round of nested-PCR. Thermocycling conditions for the 2nd round of nested-PCR consisted of 1 cycle at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s; 55 °C for 30 s; 72 °C for 1 min; a final extension cycle (72 °C for 5 min), and finally a hold at 4 °C. The PCR products of 2nd nested-PCR were detected by agarose gel (2%) electrophoresis. Positive samples were purified using QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's guidelines. The purified products were sent to Uppsala Genome Center for capillary electrophoresis sequencing using the nested primers. All in-house primers for the 1st round and the 2nd round nested-PCR are given in Table 1. The same primer sequences were also used by us in an another study (Danielsson et al., 2013, Manuscript).

2.3. Mutation analysis

The HCV NS3 sequences were analyzed using SeqScape® Software v2.6 (Applied Biosystems). We used the NS3 sequence of HCV genotype 1a H77 strain for reference template, as recommended for genotype 1 by the HCV drug development advisory group meeting 2011 (HCV DrAG) (Kwong et al., 2011). To simplify, we considered this reference template suitable for other genotypes as well. The mutation sites were noted manually. For interpretation of patient samples, the fold resistance data for particular drugs were taken from the literature sources mainly through HCV DrAG

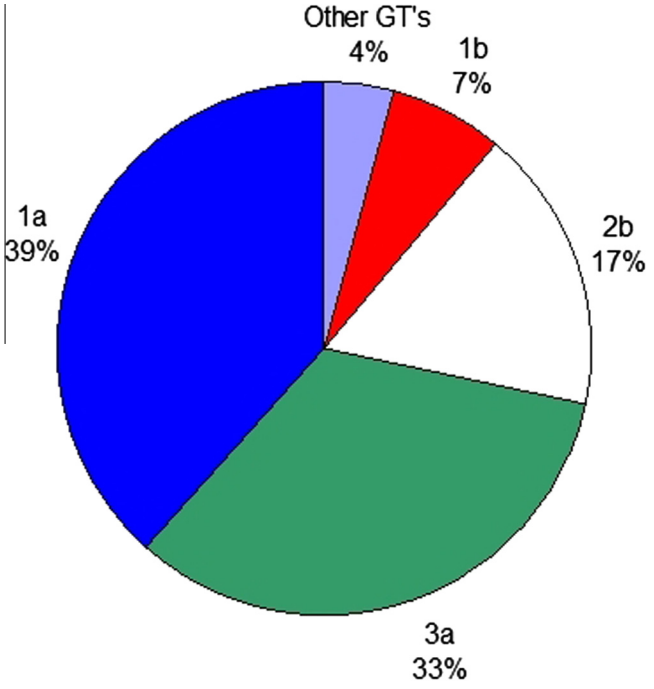

Fig. 1. Distribution of HCV genotypes based on 872 patients that were analysed during the year 2008–2012 in the Uppsala–Gävle–Örebro region.

2012 (HCV Phenotype Working Group and HCV Drug Development Advisory Group, 2012). Thus mutations/polymorphisms considered as potential RAVs (defined as difference from genotype 1a reference sequence H77) in this study are found at codon positions, 36, 41, 43, 54, 55, 80, 122, 155, 156, 168 and 170. Thereby, we attempt to associate phenotype data from genotype 1 to other genotypes.

3. Results

3.1. Distribution of HCV genotypes in the study

All of the 99 samples had been HCV genotyped prior to collection. The HCV genotype distribution was 1a in 53.5% (53/99), genotype 3a in 30.3% (30/99), genotypes 2b and 1b were found in a smaller number of patients constituting 11.1% (11/99) and 5.1% (5/99), respectively. These percentages are close to the normal distribution of genotypes in the Uppsala region 2008–2012 (Fig. 1).

3.2. Mutations/polymorphisms in HCV NS3 protease gene in samples from PI treatment-naive patients with respect to individual genotypes

The prevalence of PI RAVs in genotype 1a samples was found to be 28% (15/53) (Table 2). Mutations V36L, T54A, T54S, V55A, V55I, Q80K and Q80R were found in 3.8, 1.9, 7.5, 7.5, 5.7, 5.7 and 3.8% of genotype 1a patients, respectively. Nearly 7.5% (4/53) of the genotype 1a patient samples had more than 1 PI RAVs, i.e. three had T54S and V55I, and one had T54A and V55A. No PI RAVs were found in the five genotype 1b samples.

The material included a total of 11 HCV genotype 2b patient samples. All of them (100%) had multiple PI RAV related polymorphisms: V36L, Q80G and S122R. Specific mutation like T54S was found in 9.1% (1/11) of genotype 2b patients.

HCV genotype 3a was seen in 30 patient samples. In all of them, we found multiple PI RAVs like V36L, and D168Q. We did not find any other PI RAV related polymorphisms in genotype 3a samples.

We did not discover any other potential RAVs in the genotype 1a, 1b, 2b and 3a samples, i.e. at codon positions 41(Q), 43(F), 155(R), 156(A) and 170(I/V) that differed from the genotype 1a H77 strain. The same polymorphisms at these positions were observed when H77 was compared with genotype 2b reference strain AB030907 and genotype 3a strain D17763.

3.3. Interpretation of RAVs and polymorphism according to the literature for fold-change of phenotypic resistance

We interpreted our findings using the *in vitro* fold resistance data found in the literature for HCV NS3 protease gene mutations associated with reductions in susceptibility to the two 1st generation PIs (boceprevir and telaprevir) and two 2nd generation agents (faldaprevir and simeprevir) (Table 2). In our study, 3.8% of genotype 1a, 100% of genotype 2b and 100% of genotype 3a had a specific mutation V36L. According to the literature, this mutation confers reduced susceptibility to telaprevir with a 3.1-fold increase in IC_{50} when compared with the wild type in a replicon assay (Lenz et al., 2010). Similarly, 7.5% of genotype 1a patients had a specific mutation T54S. This mutation confers resistance to faldaprevir with 3.5-fold increase in IC_{50} (Lagace et al., 2012). Further, the PI RAVs in genotype 1a were quite diverse in comparison with other

Table 2
Fold resistance against PIs for the studied patient samples.

Mutations	Genotype						Fold resistance data from literatures[r]			
	1a (N = 53)		2b (N = 11)		3a (N = 30)		Boceprevir[t] (SCH-503034)	Telaprevir[r] (VX-950)	Faldaprevir[u] (BI-201335)	Simeprevir[t,v] (TMC-435)
	n	%	n	%	n	%				
V36L	2	3.8	11	100	30	100	1.6	3.1	NDA	1.7
T54A[w]	1	1.9	0	0	0	0	2.1	7.5	0.9	0.6
T54S[w]	4	7.5	1	9.1	0	0	8.5	NDA	3.5	1.2
V55A[w]	4	7.5	0	0	0	0	6.9[s]	NDA	NDA	NDA
V55I[w]	3	5.7	0	0	0	0	NDA	NDA	NDA	NDA
Q80G	0	0	11	100	0	0	1.2	1.2	NDA	1.8
Q80K	3	5.7	0	0	0	0	0.8	0.5	2.2	7.7
Q80R	2	3.8	0	0	0	0	0.5	0.6	2.6	6.9
S122R	0	0	11	100	0	0	NDA	NDA	NDA	20
D168Q	0	0	0	0	30	100	NDA	NDA	NDA	700

NDA = No data available about fold resistance.
[r] Based on replicon assays with genotype 1a or genotype 1b. The fold resistance values are in comparison with the wild type.
[s] Given by (Susser et al., 2009).
[t] Given by (Lenz et al., 2010).
[u] Given by (Lagace et al., 2012).
[v] Given by (Lenz et al., 2012b).
[w] Three genotype 1a samples had double mutation T54S and V55I and one 1a sample had double mutation T54A and V55A.

genotypes in our study. One genotype 1a had mutation T54A. This mutation confers resistance to multiple drugs (i.e. both boceprevir and telaprevir) with 2.1 and 7.5-fold increase in IC_{50}, respectively (Lenz et al., 2010). Mutation V55A tends to confer a 6.9-fold reduced susceptibility to boceprevir (Susser et al., 2009), we found 7.5% of genotype 1a with this mutation. Mutation Q80 K/R confers between 2–7-fold resistance to both faldaprevir and simeprevir (Lagace et al., 2012; Lenz et al., 2010), and in our study, five genotype 1a samples were found as three Q80K and two Q80R.

In contrast to the relatively low levels of resistance for RAVs in genotype 1a, certain polymorphisms namely, S122R in genotype 2b and D168Q in 3a, stand out. In studies with replicon assay of the S122R and D168Q, as site directed mutations in genotype 1b background, conferred 20 and 700 fold resistance to simeprevir, respectively (Lenz et al., 2012b).

4. Discussion

The introduction of DAAs along with the pegylated-interferon-α plus ribavirin regimen to treat HCV genotype 1 infections is a landmark for HCV treatment. PIs rapidly reduce the viral load in most of the treated genotype 1 patients, the sustained viral response (SVR) rates increase, and the length of treatment may often be reduced in comparison with the old SOC regimen (Bacon et al., 2011; Zeuzem et al., 2011). However, there is a risk for development of resistance against the DAAs. A study demonstrated that, when HCV genotype 1a was treated with telaprevir as monotherapy, within 14 days, 100% of the variants isolated from the patients showed resistance to that drug (Susser et al., 2011). It is well known that many mutations involved in the resistance to PIs have a diminishing effect on viral fitness (He et al., 2008). However, some substitutions have less critical fitness effect, e.g. when placed further from the protease catalytical site. Subsequently, naturally occurring mutations that confers reduced susceptibility have been found occasionally in patients even prior to the start of treatment with DAAs (Bartels et al., 2008, 2013; Kuntzen et al., 2008). These studies only included genotype 1 patients. However, other studies of natural resistance to PIs also included genotype 3a besides 1a and 1b (Gaudieri et al., 2009), and lately prevalence of PI RAVs have been studied in genotype 1a, 1b, 2c, 3a and 4a/c/d (Paolucci et al., 2012). No previous study has included the prevalence of baseline PI RAVs in HCV-infected patients in Sweden. In this case, genotypes 1a, 2b and 3a were included, which represents 39, 17 and 33%, respectively, in the Uppsala–Gävle–Örebro region (Fig. 1). Furthermore, previous studies have not tried to fully interpret the fold resistance level associated with PI failure in such genotypes, except the study by Bartels et al. (2013), although it included only genotype 1a and 1b.

In this study, the prevalence of PI RAVs was high (28%) in the genotype 1a samples. We could not find any RAVs in the genotype 1b patient samples; this might be due to the small number of samples from such patients. As seen in Table 2, most of the baseline RAVs found in genotype 1a confers relative low level of resistance (2–3-fold change) which could be considered within the error range of the replicon assays. However, some of the 54, 55 and 80 variations render 7–8-fold level to either boceprevir, telaprevir or simeprevir. Four genotype 1a patients had mutations at two codon positions in the viral NS3 protease gene; T54S + V55I (in three patients) and T54A + V55A (in one patient). The combination of these low level boceprevir/telaprevir resistant mutations has been observed by others and does not seem to additionally increase resistance to PIs, probably due to joint steric interaction by these residues (Welsch et al., 2012).

All genotype 2b samples had specific mutations like V36L, Q80G and S122R. They may represent the natural polymorphisms that distinguish genotype 2b from the rest. The mutations V36L and Q80G provides insignificant level of resistance against PIs (HCV Phenotype Working Group and HCV Drug Development Advisory Group, 2012). It has lately been confirmed that S122R mutation appears along with R155K mutation in genotype 1a patients who are failing simeprevir monotherapy (Lenz et al., 2012a). Furthermore, recent *in vitro* studies displays that the S122R mutation renders 20-fold resistance to simeprevir (Lenz et al., 2012b). This may be considered a novel finding to explain genotype 2b inherent PI drug resistance. It has been confirmed in a study of the same position but in genotype 2a, where K122 instead of R122 was found in 2a (Chan et al., 2012). In this study with site directed mutagenesis at position K122S on genotype 2a in a 2a-JFH1 replicon, i.e. when reverting it to the genotype 1 wild type, an enhanced susceptibility to PIs was observed, which should have the same consequence as reverting genotype 2b; R122S.

All genotype 3a samples had specific mutations like V36L and D168Q. They may represent the natural polymorphisms unique to genotype 3a. Amino acid change at codon position 168 from D to A/E/G/H/I/N/T/V/Y has been well studied for its resistance against faldaprevir, simeprevir and vaniprevir, resulting in 50–1000 fold resistance (Lagace et al., 2012; Lenz et al., 2010). Lately, it has been confirmed, with *in vitro* experiments using site directed mutagenesis of a genotype 1b clone, that D168Q mutation renders a 700 fold resistance to simeprevir (Lenz et al., 2012b). This probably explains why no 2nd generation PI candidates are effective against genotype 3a.

One should bear in mind that there could be other amino acid changes at unknown positions in genotype 2b and 3a that also confer resistance to these PIs (Lennerstrand et al., 2009). In addition to the Table 2 summarized through HCV DrAG 2012 (HCV Phenotype Working Group and HCV Drug Development Advisory Group, 2012), further studies with site directed mutagenesis are needed to explain why current PIs are not effective to genotype 2b and 3a. Such studies, as described above, should also be done with genotype (2 and 3) specific reference sequences, instead of using genotype 1.

One of the aims with this study was to search for the notorious R155K mutation in treatment-naive subjects. Mutation at codon position R155 (mainly R155K) confers 10–100 fold resistance to all 1st and 2nd generation PIs. This mutation is frequently found in genotype 1a treatment failure patients (HCV Phenotype Working Group and HCV Drug Development Advisory Group, 2012), and has a half life time of 1–2 years (Strahotin and Babich, 2012). Notably, a 3rd generation PI, MK-5172 in phase 2 studies has shown very promising resilience towards mutation at codon 155, including the other most known RAVs (Summa et al., 2012). The mutation K at codon 155 found in genotype 1a treatment failure patients, is an example of an amino acid change that has less diminishing effect of HCV replication capacity compared to the wild type (He et al., 2008). Other high level PI resistance mutations such as A156 V/T and D168A/V, are never observed with population sequencing method at baseline for genotype 1, since alterations at these positions probably impair the protease activity to a high degree. In a study by others, the R155K mutation was found in HCV 1a treatment-naive subjects (approx 1%) using the same type of population sequencing method as we did (Kuntzen et al., 2008; Bartels et al., 2013). It is known that mutation at R155K occurs mainly in genotype 1a because of the favourable codon shift (only one nucleotide substitution is needed) but not in genotype 1b (two needed) (Fig. 2). Similarly, we observed (with BLAST search) that genotypes 2b and 3a also need only one nucleotide substitution for the amino acid change from R to K at position 155 (Fig. 2). Thus, in combination with results from the Kuntzen et al. (2008) study and the one nucleotide switch, we expected to detect the notorious R155K in genotype 2b and 3a besides in 1a.

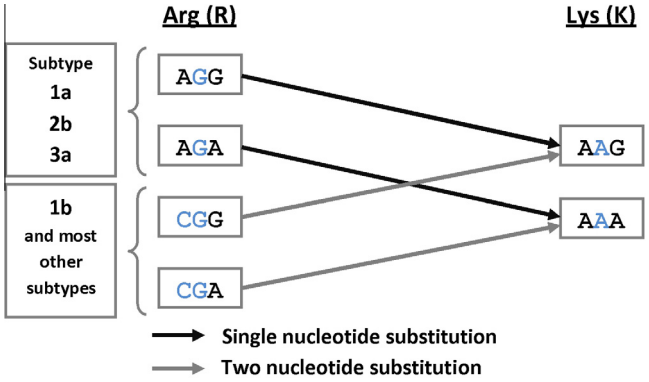

Fig. 2. R155K variants according to the genotypes. R155K is an example of an amino acid change that does not diminish the replication capacity compared to wild type. It is known that R155K mutation mainly occurs in genotype 1a treatment failure patients, because of favourable codon shift. In Sweden, there is a high prevalence of genotypes 2b and 3a as well. For both these genotypes only one nucleotide substitution is needed for R155K mutation (our observation with BLAST search).

However, we did not find any natural mutation at the 155 position in 53 genotype1a, 5 genotype 1b, 11 genotype 2b, and 30 genotype 3a samples. The reason may be the limited number of samples and that the population sequencing method only bear a 20–25% detection limit of mixes.

In the study by Bartels et al. (2013), treatment outcome was examined together with pre-existing PI mutation for more than 2000 genotype 1a patients. It was observed that patients with baseline R155K in general had an unfavourable less decline of the HCV RNA level, compared to subjects with no baseline RAVs, after four weeks therapy with SOC plus telaprevir (triple therapy) (Bartels et al., 2013). The patients with baseline RAVs V36M and T54S also showed a less decrease of RNA level, but not to the same extent as those with R155K, during triple treatment. However, it is difficult to predict treatment outcome based on baseline PI RAVs, since even the patients with major resistant variants often achieve SVR. This as triple therapy is mainly linked to activity of interferon, which activity varies depending on host genetics (IL28B status etc.). In the study by Bartels et al. (2013), of the 2000 1a subjects, they found 18 with pre-existing R155K, whereas three of these were found to be prior non responders to interferon, and neither of the three obtained SVR (Bartels et al., 2013). For such small percentage (3/2000) of genotype 1a patients to predict non-SVR, this does not motivate the use of baseline sequencing to accurately predict the efficacy of treatment with current triple therapy. However, when more DAA drug classes are available and used for all genotypes, baseline RAVs may be of interest to predict individual's optimal combination of DAAs in non-interferon regimes. Further information is needed of phenotypic susceptibility data between genotypes.

To conclude, PI RAVs were found in 28% of PI treatment naive patients infected with HCV genotype 1a. All patients with HCV genotype 2b had amino acid change patterns at positions V36, Q80 and S122, and all genotype 3a patients at positions V36 and D168. The baseline RAVs found in genotype 1a have relatively weak resistance to the approved and the 2nd generation NS3 inhibitors. However, the natural polymorphisms in genotype 2b (i.e. S122R) and 3a (i.e. D168Q), may have resistance levels against a 2nd generation NS3 inhibitor of up to 20- and 700-fold respectively in comparison with a wild type genotype 1b strain. Our preliminary results indicate that further evaluation is needed to study the role of baseline RAVs in non-genotype 1 for future interferon-free combo DAA treatments.

Acknowledgements

This study was financed by the Uppsala–Örebro Regional Research Council, Scandinavian Society for Antimicrobial Chemotherapy, and FMM of the Swedish Society for Medicine.

References

Bacon, B.R., Gordon, S.C., Lawitz, E., Marcellin, P., Vierling, J.M., Zeuzem, S., Poordad, F., Goodman, Z.D., Sings, H.L., Boparai, N., Burroughs, M., Brass, C.A., Albrecht, J.K., Esteban, R., 2011. Boceprevir for previously treated chronic HCV genotype 1 infection. N. Engl. J. Med. 364, 1207–1217.

Bartels, D.J., Zhou, Y., Zhang, E.Z., Marcial, M., Byrn, R.A., Pfeiffer, T., Tigges, A.M., Adiwijaya, B.S., Lin, C., Kwong, A.D., Kieffer, T.L., 2008. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3·4A protease inhibitors in treatment-naive subjects. J. Infect. Dis. 198, 800–807.

Bartels, D.J., Sullivan, J.C., Zhang, E.Z., Tigges, A.M., Dorrian, J.L., Meyer, S.D., Takemoto, D., Dondero, E., Kwong, A.D., Picchio, G., Kieffer, T.L., 2013. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J. Virol. 87, 1544–1553.

Chan, K., Peng, B., Chen, X., Appleby, T.C., Taylor, J., Delaney, W.E., Cheng, G., 2012. Influence of hepatitis C virus genotype 2 subtype and NS3 polymorphisms on protease inhibitor antiviral activity. Poster 1077 Presented at the 63rd annual meeting of the AASLD, Boston, United States.

Danielsson, A., Palanisamy, N., Golbob, S., Yin, H., Blomberg, J., Hedlund, J., Sylvan, S., Lennerstrand, J., 2013. Transmission of hepatitis C virus among intravenous drug users in the Uppsala region of Sweden. Manuscript.

Duberg, A., Janzon, R., Back, E., Ekdahl, K., Blaxhult, A., 2008. The epidemiology of hepatitis C virus infection in Sweden. Euro. Surveill. 13, pii=18882.

Gaudieri, S., Rauch, A., Pfafferott, K., Barnes, E., Cheng, W., McCaughan, G., Shackel, N., Jeffrey, G.P., Mollison, L., Baker, R., Furrer, H., Gunthard, H.F., Freitas, E., Humphreys, I., Klenerman, P., Mallal, S., James, I., Roberts, S., Nolan, D., Lucas, M., 2009. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. Hepatology 49, 1069–1082.

Gravitz, L., 2011. Introduction: a smouldering public-health crisis. Nature 474, S2–S4.

Hadziyannis, S.J., Sette, J.H., Morgan, T.R., Balan, V., Diago, M., Marcellin, P., Ramadori, G., Bodenheimer, J.H., Bernstein, D., Rizzetto, M., Zeuzem, S., Pockros, P.J., Lin, A., Ackrill, A.M., 2004. Peginterferon-α2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann. Intern. Med. 140, 346–355.

Hagan, L.M., Schinazi, R.F., 2013. Best strategies for global HCV eradication. Liver Int. 33, S68–S79.

HCV phenotype working group, HCV drug development advisory group, 2012. Clinically relevant HCV drug resistance mutations figure and tables. Ann. Forum Collab. HIV Res. 14 (2), 1–10.

He, Y., King, M.S., Kempf, D.J., Lu, L., Lim, H.B., Krishnan, P., Kati, W., Middleton, T., Molla, A., 2008. Relative replication capacity and selective advantage profiles of protease inhibitor-resistant hepatitis C virus (HCV) NS3 protease mutants in the HCV genotype 1b replicon system. Antimicrob. Agents Chemother. 52, 1101–1110.

Kowdley, K.V., 2005. Hematologic side effects of interferon and ribavirin therapy. J. Clin. Gastroenterol. 39, S3–S8.

Kuntzen, T., Timm, J., Berical, A., Lennon, N., Berlin, A.M., Young, S.K., Lee, B., Heckerman, D., Carlson, J., Reyor, L.L., Kleyman, M., McMahon, C.M., Birch, C., Schulze zur Wiesch, J., Ledlie, T., Koehrsen, M., Kodira, C., Roberts, A.D., Lauer, G.M., Rosen, H.R., Bihl, F., Cerny, A., Spengler, U., Liu, Z., Kim, A.Y., Xing, Y., Schneidewind, A., Madey, M.A., Fleckenstein, J.F., Park, V.M., Galagan, J.E., Nusbaum, C., Walker, B.D., Lake-Bakaar, G.V., Daar, E.S., Jacobson, I.M., Gomperts, E.D., Edlin, B.R., Donfield, S.M., Chung, R.T., Talal, A.H., Marion, T., Birren, B.W., Henn, M.R., Allen, T.M., 2008. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naïve patients. Hepatology 48, 1769–1778.

Kwong, A.D., Najera, I., Bechtel, J., Bowden, S., Fitzgibbon, J., Harrington, P., Kempf, D., Kieffer, T.L., Koletzki, D., Kukolj, G., Lim, S., Pilot-Matias, T., Lin, K., Mani, N., Mo, H., O'Rear, J., Otto, M., Parkin, N., Pawlotsky, J.M., Petropoulos, C., Picchio, G., Ralston, R., Reeves, J.D., Schooley, R.T., Seiwert, S., Standring, D., Stuyver, L., Sullivan, J., Miller, V., 2011. Sequence and phenotypic analysis for resistance monitoring in hepatitis C virus drug development: recommendations from the HCV DRAG. Gastroenterology 140, 755–760.

Lagace, L., White, P.W., Bousquet, C., Dansereau, N., Do, F., Llinas-Brunet, M., Marquis, M., Massariol, M.J., Maurice, R., Spickler, C., Thibeault, D., Triki, I., Zhao, S., Kukolj, G., 2012. *In vitro* resistance profile of the hepatitis C virus NS3 protease inhibitor BI 201335. Antimicrob. Agents Chemother. 56, 569–572.

Lennerstrand, J., Bondeson, K., Bergqvist, A., Blomberg, J., Oberg, B., 2009. New antiviral agents against hepatitis C in clinical trials. Hope for a cure-but resistance problems must be overcomed. Lakartidningen 106, 3254–3256, 3258, 3260.

Lenz, O., Verbinnen, T., Lin, T.I., Vijgen, L., Cummings, M.D., Lindberg, J., Berke, J.M., Dehertogh, P., Fransen, E., Scholliers, A., Vermeiren, K., Ivens, T., Raboisson, P., Edlund, M., Storm, S., Vrang, L., de Kock, H., Fanning, G.C., Simmen, K.A., 2010. *In vitro* resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob. Agents Chemother. 54, 1878–1887.

Lenz, O., de Bruijne, J., Vijgen, L., Verbinnen, T., Weegink, C., Van Marck, H., Vandenbroucke, I., Peeters, M., Simmen, K., Fanning, G., Verloes, R., Picchio, G., Reesink, H., 2012a. Efficacy of re-treatment with TMC435 as combination therapy in hepatitis C virus-infected patients following TMC435 monotherapy. Gastroenterology 143, 1176–1178.

Lenz, O., Vijgen, L., Berke, J.M., Cummings, M.D., Fevery, B., Peeters, M., Smedt, G.D., Moreno, C., Picchio, G., 2012b. Virologic response and characterization of HCV genotypes 2–6 in patients receiving TMC435 monotherapy (study TMC435-C202). J. Hepatol.. http://dx.doi.org/10.1016/j.jhep.2012.10.028.

Lin, C., Lin, K., Luong, Y.P., Rao, B.G., Wei, Y.Y., Brennan, D.L., Fulghum, J.R., Hsiao, H.M., Ma, S., Maxwell, J.P., Cottrell, K.M., Perni, R.B., Gates, C.A., Kwong, A.D., 2004. *In vitro* resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J. Biol. Chem. 279, 17508–17514.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M.H., Albrecht, J.K., 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358, 958–965.

Månsson, A.S., Moestrup, T., Nordenfelt, E., Widell, A., 2000. Continued transmission of hepatitis B and C viruses, but no transmission of human immunodeficiency virus among intravenous drug users participating in a syringe/needle exchange program. Scand. J. Infect. Dis. 32, 253–258.

Martell, M., Esteban, J.I., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J., Gomez, J., 1992. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J. Virol. 66, 3225–3229.

Nakano, T., Lau, G.M.G., Lau, G.M.L., Sugiyama, M., Mizokami, M., 2012. An updated analysis of hepatitis C virus genotypes and subtypes based on the complete coding region. Liver Int. 32, 339–345.

Norda, R., Duberg, A.S., Sönnerborg, A., Olcén, P., 1995. Transmission of hepatitis C virus by transfusion in Örebro county, Sweden, 1990–1992. Scand. J. Infect. Dis. 27, 449–452.

Paolucci, S., Fiorina, L., Piralla, A., Gulminetti, R., Novati, S., Barbarini, G., Sacchi, P., Gatti, M., Dossena, L., Baldanti, F., 2012. Naturally occurring mutations to HCV protease inhibitors in treatment-naïve patients. Virol. J. 9, 245. http://dx.doi.org/10.1186/1743-422X-9-245.

Poordad, F., Chee, G., 2012. Interferon free hepatitis C treatment regimens: the beginning of another era. Curr. Gastroenterol. Rep. 14, 74–77.

Prati, D., 2006. Transmission of hepatitis C virus by blood transfusions and other medical procedures: a global review. J. Hepatol. 45, 607–616.

Shepard, C.W., Finelli, L., Alter, M.J., 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect. Dis. 5, 558–567.

Shev, S., Hermodsson, S., Lindholm, A., Malm, E., Widell, A., Norkrans, G., 1995. Risk factor exposure among hepatitis C virus RNA positive Swedish blood donors – the role of parenteral and sexual transmission. Scand. J. Infect. Dis. 27, 99–104.

Soriano, V., Perelson, A.S., Zoulim, F., 2008. Why are there different dynamics in the selection of drug resistance in HIV and hepatitis B and C viruses? J. Antimicrob. Chemother. 62, 1–4.

Soriano, V., Vispo, E., Poveda, E., Labarga, P., Martin-Carbonero, L., Fernandez-Montero, J.V., Barreiro, P., 2011. Directly acting antivirals against hepatitis C virus. J. Antimicrob. Chemother. 66, 1673–1686.

Strahotin, C.S., Babich, M., 2012. Hepatitis C variability, patterns of resistance, and impact on therapy. Adv. Virol. 2012, 1–10.

Summa, V., Ludmerer, S.W., McCauley, J.A., Fandozzi, C., Burlein, C., Claudio, G., Coleman, P.J., DiMuzio, J.M., Ferrara, M., Di Filippo, M., Gates, A.T., Graham, D.J., Harper, S., Hazuda, D.J., McHale, C., Monteagudo, E., Pucci, V., Rowley, M., Rudd, M.T., Soriano, A., Stahlhut, M.W., Vacca, J.P., Olsen, D.B., Liverton, N.J., Carroll, S.S., 2012. MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. Antimicrob. Agents Chemother. 56, 4161–4167.

Susser, S., Welsch, C., Wang, Y., Zettler, M., Domingues, F.S., Karey, U., Hughes, E., Ralston, R., Tong, X., Herrmann, E., Zeuzem, S., Sarrazin, C., 2009. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. Hepatology 50, 1709–1718.

Susser, S., Vermehren, J., Forestier, N., Welker, M.W., Grigorian, N., Füller, C., Perner, D., Zeuzem, S., Sarrazin, C., 2011. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J. Clin. Virol. 52, 321–327.

Te, H.S., Jensen, D.M., 2010. Epidemiology of hepatitis B and C viruses: a global overview. Clin. Liver Dis. 14, 1–21.

Thomas, X.V., de Bruijne, J., Sullivan, J.C., Kieffer, T.L., Ho, C.K.Y., Rebers, S.P., de Vries, M., Reesink, H.W., Weegink, C.J., Molenkamp, R., Schinkel, J., 2012. Evaluation of persistence of resistant variants with ultra-deep pyrosequencing in chronic hepatitis C patients treated with telaprevir. PLoS ONE 7, e41191.

Welsch, C., Schweizer, S., Shimakami, T., Domingues, F.S., Kim, S., Lemon, S.M., Antes, I., 2012. Ketoamide resistance and hepatitis C virus fitness in Val55 variants of the NS3 serine protease. Antimicrob. Agents Chemother. 56, 1907–1915.

Zeuzem, S., Andreone, P., Pol, S., Lawitz, E., Diago, M., Roberts, S., Focaccia, R., Younossi, Z., Foster, G.R., Horban, A., Ferenci, P., Nevens, F., Müllhaupt, B., Pockros, P., Terg, R., Shouval, D., van Hoek, B., Weiland, O., Van Heeswijk, R., De Meyer, S., Luo, D., Boogaerts, G., Polo, R., Picchio, G., Beumont, M., 2011. Telaprevir for retreatment of HCV infection. N. Engl. J. Med. 364, 2417–2428.