A patient classified as HCV-1a-positive by both LiPA Siemens 2.0 and Abbott RealTime HCV Genotype II was instead found to be infected with HCV-1g, as determined by phylogenetic analysis of NS3 sequences. HCV-1g NS3 sequences available to date naturally harbour the resistance substitution T54S, plus P131S and L135F changes, located in the highly conserved NS3 positions within the boceprevir-binding site, as determined by structural modelling. HCV-1g NS3 sequences show some similarities to HCV-4 and are poorly responsive to interferon/ribavirin and to boceprevir/telaprevir; this patient was also a null-responder to boceprevir treatment. Baseline genotypic resistance testing may provide crucial information for the management of first-generation protease-inhibitor-based regimens, including both HCV genotype/subtype and natural resistance.

The use of HCV protease inhibitors (PIs) telaprevir and boceprevir in clinical practice has recently been approved only for the treatment of patients infected with HCV genotype 1 [1]. The determination of HCV genotype is thus mandatory prior to treatment initiation [1] and different commercial assays are, to date, available to accurately identify the most common six HCV genotypes [2]. Within the HCV genotype-1, the most common subtypes, HCV-1a and HCV-1b, have shown different responses to PI-based regimens. Indeed, both selection of resistant variants and viral breakthrough were observed consistently more frequently in patients infected with HCV-1a than HCV-1b [3–7], probably due to a different genetic barrier towards the development of resistance in HCV-1a [8–11]. HCV subtyping may thus help to select future treatment regimens and predict the development of resistance. So far, currently used commercial assays targeting the 5′-non-coding region of the HCV genome fail to efficiently differentiate HCV subtypes 1a and 1b in a substantial proportion of patients [12]. Furthermore, several other HCV-1 subtypes have been recently described to circulate in the worldwide population [13], and a number of partial sequences from subtypes 1c to 1m and unassigned subtypes are registered in both GenBank and Los Alamos HCV sequence databases [13]. As already shown for HCV-1a and HCV-1b, these also ‘uncommon’ HCV-1 subtypes can be misclassified by currently used commercial HCV genotyping assays, and can harbour different profiles of response to PI-based treatment, thus potentially affecting the efficacy of new treatment strategies.

In the context of a PI treatment monitoring study where 18 HCV-1-infected patients were treated with boceprevir plus pegylated interferon (PEG-IFN) and ribavirin [14], at week 12 of treatment two patients had HCV RNA >100 IU/ml by Abbott RealTime (detection limit =12 IU/ml; Abbott Laboratories, Abbott Park, IL, USA) and consequently interrupted therapy according to guidelines [1]. One of the two patients was a non-responder HCV-1b patient who developed the major resistance variant T54A after 8 weeks of treatment, with HCV RNA of 5.2 log10 IU/ml. The second patient was an Egyptian patient classified as infected with HCV-1a by the LiPA
VERSANT® HCV Genotype 2.0 (Siemens AG, Erlangen, Germany), a second-generation assay containing probes targeting both the 5’-non-coding and the core regions of the viral genome. He was male, 40 years old, HCV-positive since 2003 and without evidence of other viral coinfections or liver cirrhosis. Baseline HCV RNA was 6.6 log10 IU/ml and, during the lead-in phase, HCV RNA decay was extremely low (0.5 log10 IU/ml), concordant with an unfavourable IL28B genotype (TT) and a history of null-response to previous PEG-IFN-α2b plus ribavirin treatments. HCV RNA was 5.7 log10 IU/ml at both 8 and 12 weeks (that is, 4 and 8 weeks of boceprevir, respectively) with an overall viraemia decay less than 1 log10 IU/ml, thus classifiable as a null-response. NS3 sequences encompassing the protease domain (amino acids 1–181) were obtained at baseline (T0) and at weeks 8 (W8) and 12 (W12) after starting treatment (Figure 1A).

The baseline analysis of NS3 sequences revealed the presence of the boceprevir-resistance-associated amino acid substitution T54S. The T54S has been previously found to develop under boceprevir treatment in vivo [6] and to reduce viral sensitivity to boceprevir treatment [15]. Notably, during boceprevir treatment and failure, no additional NS3 protease mutations were developed in this patient. Given his high compliance, this suggests that the mutations present at baseline were sufficient to confer resistance to the triple therapy.

The baseline presence of T54S was found also in another boceprevir-treated patient included in this PI treatment monitoring study [14], causing a delay in virological response. This last patient achieved undetectable HCV RNA at week 16, but she stopped boceprevir treatment due to the onset of a severe anaemia. Therefore, the effect of the baseline T54S variant on virological outcome could not be assessed in this case.

Along with T54S substitution, several other variations compared with HCV-1a reference strain (GenBank accession number M62321) were detected in NS3 sequences at all analysed time points of the null-responder patient. This consistent presence of mutations prompted us to perform a phylogenetic analysis to confirm the HCV genotype. A maximum likelihood phylogenetic tree was inferred by MEGA5 software, using a general time reversible model for nucleotide substitutions and applying a gamma distribution with invariant sites (4 rate categories). Phylogenetic analysis was performed including all HCV genotypes (≤ 0.1% amino acid variability) [8].

The phylogenetic analysis of NS3 sequences obtained at baseline, at weeks 8 and 12 were closely related to HCV-1g reference (Figure 1A) and formed a distinct cluster from other HCV-1 subtypes, thus indicating that the infecting HCV strain is HCV-1g. HCV-1g is a recognized HCV-1 subtype, previously identified in Spain and Egypt [16,17], for which unfortunately only one NS3 sequence is available today in GenBank.

The phylogenetic analysis was confirmed by an evolutionary divergence analysis, performed with MEGA5 software applying the Kimura 2-parameter model and a gamma distribution (shape parameter = 1). Indeed, the estimated nucleotide evolutionary divergence was found to be higher between HCV-1g and the other HCV-1 subtypes (median [IQR] 0.402 [0.387–0.458] number of substitutions/sites) compared with what was observed among HCV-1a/1b/1c (median [IQR] 0.280 [0.269–0.341] number of substitutions/sites). The median (IQR) estimated nucleotide evolutionary divergence among HCV-1g sequences was 0.07 (0.07–0.08) number of substitutions/site. Furthermore, the evolutionary divergence analysis indicated that, besides HCV-1a/b/c, the most similar HCV subtype was HCV-4b (evolutionary divergence = 0.502 ± 0.057 number of substitutions/site), usually found among Egyptian patients (such as our one). This genetic similarity is interesting from a clinical point of view because HCV-4 is poorly sensitive to interferon/ribavirin and to first-generation PI treatment.

There are no data on the sensitivity of HCV-1g strains to PI-based treatment and the non-response to boceprevir treatment could be related to the uncommon genetic backbone observed in HCV-1g viral strain. Indeed, the T54S was found in both HCV-1g sequences available today (one available in GenBank and one obtained in the present study), indicating a possible wild-type presence of this substitution in HCV-1g. Furthermore, 25/181 and 20/181 NS3 residues differed, respectively, between the HCV-1g patient’s baseline sequence in comparison to HCV-1a and HCV-1b reference sequences (Figure 1B). Compared with HCV-1g reference AM910652, our sequences showed the F63T and P86Q amino acid substitutions at all time points. Interestingly, HCV-1g sequences presented two specific amino acid changes, P131S and L135F, located in highly conserved NS3 positions among all HCV genotypes (≤ 0.1% amino acid variability) [8]. These two residues are located near the enzyme’s active site and the boceprevir binding site, as determined by structural modelling of NS3-1a three-dimensional structure (PDB ID: 3LOX) complexed with a derivative of boceprevir (Figure 2). These variations can thus suggest a different three-dimensional conformation of the enzyme, which may impair the formation of proper contact with the drug, also considering that residue 135 is one of the NS3 residues interacting with boceprevir [8]. Therefore, the role of P131S and L135F variants, in presence and absence of T54S, on the sensitivity to boceprevir treatment deserves further investigations and both phenotypic assays and additional in vivo data are required to confirm their association with PI resistance.
Notably, when the baseline sample was re-tested for HCV genotype with Abbott RealTime HCV-Genotype II (m2000 system; Abbott Laboratories), the patient was again classified as infected with HCV-1a, thus confirming the incorrect result previously obtained by LiPA VERSANT Siemens 2.0 and indicating the difficulty of the next-generation diagnostic tools to discriminate HCV-1g sequences.

Along with literature data [3–7], this case underlines the importance of HCV subtype in determining the success of PI-based therapies, highlighting the circulation, in specific geographic areas, of uncommon HCV-1 subtypes potentially insensitive to PI treatment and not recognized by currently available commercial HCV genotyping assays [12].

In the context of a PI regimen, the performance of baseline sequencing of HCV can thus fulfill, at the same time, two important requirements for clinical management of patients with chronic HCV infection: a correct subtype assignment and detection of variants that are potential non-responders to therapy [18–21]. Overall, the cost-effectiveness of HCV sequencing may deserve

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Figure 1. Genetic diversity of HCV-1g subtype determined by a maximum likelihood phylogenetic tree of NS3 protease sequences and an NS3 protease amino acid sequence comparison

**A**

![Phylogenetic Tree](image)

**B**

<table>
<thead>
<tr>
<th>NS3 protease amino acid position</th>
<th>14 18 26 28 40 48 52 54 63 64 66 67 72 86 89 91 101 114 125 131 132 135 146 150 151 170 174</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV-1a M62321</td>
<td>L I K Q T I C T T I S P I P Q S S I S P I L P A A V I N</td>
</tr>
<tr>
<td>HCV-1b D90208 sequence</td>
<td>- - - - - V - - - L G - T - P A - - - - V - - - - S - - V S</td>
</tr>
<tr>
<td>HCV-1g AM910652 sequence</td>
<td>M V N T N V L S F L A R A - A A A V A S V F S S V A V S</td>
</tr>
<tr>
<td>HCV-1g PT14_T0 sequence</td>
<td>M V N T N V L S - L A R A Q A A A V A S V F S S V A V S</td>
</tr>
</tbody>
</table>

Genetic diversity of HCV-1g subtype determined by a maximum likelihood phylogenetic tree of (A) NS3 protease sequences and (B) an NS3 protease amino acid sequence comparison. The tree was inferred using a GTR+G+I model for nucleotide substitutions. Numbers above branches represent bootstrap values (1,000 replicates). Only values >70 are reported. The patient's sequences at three different time points during treatment (baseline [T0], week 8 [W8] and week 12 [W12]) are reported in bold. Reference sequences are reported with the relative GenBank accession number.
Figure 2. NS3-1a three-dimensional structure® complexed with a derivative of boceprevir® modified with USCF Chimera 1.5.3 software®

9PDB_ID: 3LOX. *In black. (22) Residues 131 and 135 fully conserved among all HCV genotypes but polymorphic in HCV-1g are represented in light grey, while amino acids found mutated only in PT14 (T63 and P86) are represented in dark grey.

further attention in clinical practice because it may reveal crucial factors in assessing the probability of success with first-generation PI-based regimens.

Acknowledgements

We thank Ada Bertoli and Marco Ciotti for technical support in the management of clinical samples. This work was supported by AVIRALIA foundation, and from the Italian Ministry of Instruction, University & Research (MIUR; RBAP11YS7K_001; ‘InterOmics’ project PB.P05 coordinated by the CNR). A part of these results was presented at the International HIV & Hepatitis Virus Drug Resistance Workshop (Sitges, Spain; 5–9 June 2012, abstract 80).

Disclosure statement

The authors declare no competing interests.

References

22. UCSF Chimera 1.5.3 software. (Accessed 18 December 2012.) Available from http://www.cgl.ucsf.edu/index.html