Genotypic analysis of HCV 1a by sequencing of the NS3 proteasic region in simeprevir therapy candidates

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SUMMARY

Each phase of the HCV replication cycle can represent a therapy target. In fact, SIMEPREVIR (SMV) acts as NS3/4A protease inhibitor (PI); its efficacy is, however, reduced in HCV1a patients characterized by NS3Q80K polymorphism. The aim of this work was to design a genotypic analysis of NS3 protease in order to characterize viral quasispecies in HCV 1a patients before starting the SMV therapy. In all, 38 peripheral blood-EDTA samples were collected from patients infected with HCV 1a (RNA >10,000 cp/ml). The samples were sequenced in a region of 543 nucleotides, codifying for 181 amino acids of the NS3 protease with ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Of the 38 samples, two showed the Q80K mutation associated with resistance to SMV. In 16 samples mutations associated with a possible resistance to protease inhibitor, TELAPREVIR, were observed. Only one sample showed the T54S mutation, which is responsible for resistance to BOCEPREVIR, a protease inhibitor too. The data reported in this paper show a 5% prevalence of the Q80K mutation in HCV 1a patients. So far, some differences in the percentage of the Q80K mutations were observed within the European population, when compared with its US counterpart. The prevalence study described herein, albeit observed on a low number of samples, could challenge the recommendations reported in the technical data sheet of SMV.

Keywords: HCV, NS3 protease, Q80K, Simeprevir.

INTRODUCTION

The hepatitis C virus (HCV) infection is widely spread (≈ 3% of the world population in 2013) [1]. It represents a serious health problem, since about 80% of HCV infected population will develop a chronic and progressive disease, characterized by fibrosis and cirrhosis of the liver that in 15-20% of the cases may result in deadly hepatocarcinoma. For a long time, the chronic HCV infection was treated with pegylated interferon (PEG-IFN) in association with ribavirin (RBV) for at least 48 weeks. The low percentage of Sustained Viral Response (SVR), particularly observed in patients with 1a HCV, the length of the treatment and the onset of important adverse effects promoted studies aiming to better characterize the virus and its life cycle [2].

Studies on the life cycle and on the virus genome and protein structure have allowed outlining and introducing (since 2011) the Direct Antiviral Agents (DAA) in the clinical practice. Each phase of the life cycle can be a therapy target, and all the virus enzymes (NS2 and NS3/4 proteases, their NS4A cofactor, the NS5A replication complex and the NS5B RNA polymerase dependent) are potential targets of anti-HCV drugs [3]. The

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use of 2nd generation drugs, aiming at the rise of the genetic barrier and at the increase of the activity against other genotypes, shows promising results [4, 5].
In particular, the NS3 protein weighs 69 KDa, and possesses more functional domains. It is equipped with a domain responsible for the protease activity - located at N terminal - and a domain responsible for the RNA helicase and NTPase activity - located at the C terminal.
The non-covalent interaction between NS3 and the cofactor NS4A manages the formation of the NS3/4A serine protease which is responsible for the processing of the polyprotein precursor [6].
Simeprevir (SMV) (OLYSIO®) is a second-generation macrocyclic inhibitor of NS3/4A protease (PI). A single daily dose of 150 mg is taken with food, in association with PEG-IFN and RBV. Compared with linear first-generation PIs, which present linear structure, SMV shows a more advantageous binding affinity and specificity towards the NS3/4A protease and a lower occurrence of side effects [7]. SMV is active against all the genotypes, although it has currently been approved for the treatment of only 1a HCV patients [8].
The SMV technical data sheet reports the following recommendation: "…The effectiveness of SMV in association with PEG-IFN and RBV is substantially reduced in patients infected with 1a HCV with the NS3 Q80K polymorphism at the basal than the 1a HCV patients without the Q80K polymorphism. It is highly recommended the test for the presence of the Q80K polymorphism in 1a HCV patients considering the therapy with OLYSIO in association with PEG-IFN and RBV…"
The Q80K polymorphism is present in the protein NS3, when cytosine 238(C) of the triplet at position 80 is replaced by adenine (A); consequently, in the amino acid sequence the glutamine (Q) is replaced with lysine (K). The presence of the Q80K polymorphism significantly reduces the levels of SVR (58% to 84% in G1a treatment naïve patients; 47% to 79% in G1a patient at first relapse) [9]. It has been shown that patients with genotype 1a HCV and Q80K polymorphism who received simeprevir were statistically significantly less likely to achieve SVR than patients with genotype 1b or genotype 1a without Q80K who received simeprevir [10].

PATIENTS AND METHODS

Although the effect of Q80K on simeprevir *in vitro* activity is limited, its presence may facilitate the emergence of additional amino acid substitutions, resulting in a higher failure rate in patients treated with simeprevir plus PegIFN/RBV [11].
The aim of this work was to get ready a genotypic analysis of NS3 protease by sequencing PCR products, in order to characterize the viral quasi-species in 1aHCV patients before starting the treatment with SMV.
750 plasma samples from 1a HCV patients hospitalized at the "Cotugno Hospital" - ("Azienda Ospedaliera Specialistica dei Colli Monaldi - Cotugno - CTO", Naples, Italy) were collected from 2014 November to 2015 January, then sent to the Microbiology and Virology Laboratories of the same hospital. Thirty-eight out of the 750 samples, characterized by HCV RNA >10,000 cp/mL and genotype 1a, were selected - all coming from the "Medicina Protetta" - V.U.O.C. Department of the Cotugno Hospital Naples, Italy. The nucleic acid of the samples was quantized with the Roche Ampliprep and Taqman instrumentation, whereas their genotype was assayed with Versant HCV Genotype 2.0 kit (LiPA), which contains specific probes targeting both the 5'UTR and the Core regions of the viral genome. At the time of sampling, all patients were treatment-naïve with HCV protease inhibitors (PI). The viral RNA was extracted by hand-operated procedure, involving the cell lysis followed by separation and precipitation with isopropanol and ethanol.
Specific and suitable primers were designed before the amplification phases. The NS3 gene codifies for a protease and a helicase. The region of interest is that with protease activity consisting of 543 nucleotides codifying for 181 amino acids (Figure 1). Therefore, the reference sequence from the GenBank-accession number NC_004102 was used to construct the primers; it corresponds to the HCV strain H77 genotype 1a. Following the identification of the sequence region corresponding to the NS3 protease, sequences of 18-22 nt were selected with the highest match number on NCI BLAST nucleotide (http://blast.ncbi.nlm.nih.gov/) upstream and downstream of the above 543 nt. The primers synthesis was carried out by Tib Molbiol s.r.l.

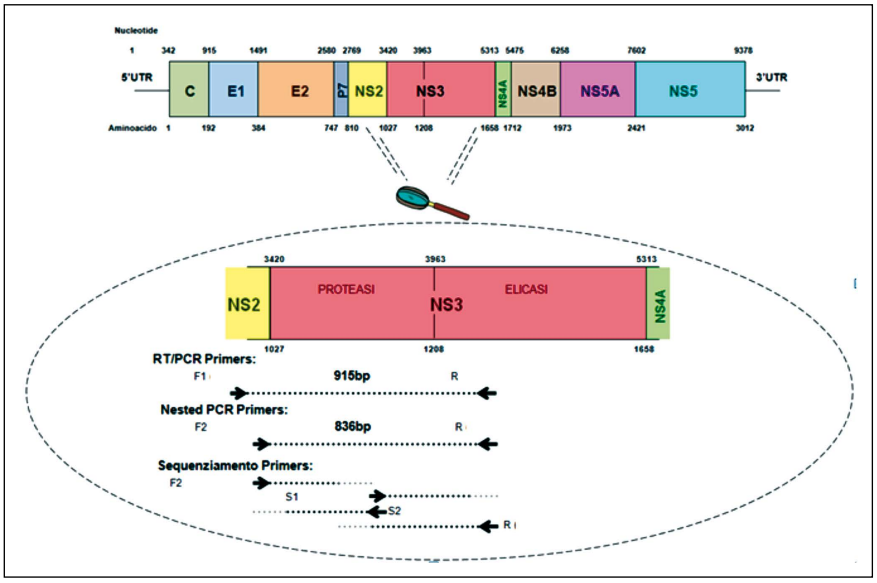

Figure 1 - Primers localization. The primers were designed having HCV H77 strain as a reference.

A fragment of 876 bp in the NS3 region of the viral genome was obtained by means of the kit SuperScript One-Step RT-PCR System (Invitrogen Corp) and the primers C1aF1 (5'-GACATCAT-CAACGGCTTGC-3') and C1aR (5'-AACTTGC-CGTAGGTGGAGT-3') at a final concentration of 0,2µM; the reaction mix was subject to the following cycles: 30' at 50°C; 2' at 94°C; 40 cycles of 30" at 94°C, 30" at 53°C, 40" at 72°C; 10' at 72°C.

If the amplification product is not evident after 1% agarose -gel electrophoresis, a semi-nested PCR must be performed.

The purification of the amplification product of the NS3 portion is followed by sequencing through BigDye terminator v.3.1-cycle-sequencing-kit (Applied Biosystems).

Each sample was distributed in four reaction tubes each containing a different primer of the following: C1aF2, C1aS1, C1aS2, C1aR. The amplification program was: 1' at 96°C; 25 cycles of 10" at 96°C, 5" at 50°C, 4' at 60°C.

The sequencing of different length fragments from the amplification process was carried out with the automatic sequencer ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The analysis and the assembly was performed with the software v2.6®" Applied Biosystem.

The nucleotide sequence in .fsta format was analysed by Geno2pheno [hcv] (http://hcv.geno2pheno.org/). This bioinformatic system compares the sample sequence with its data bank highlighting the mutations and the probable resistance profile to antiviral drugs, among them the SMV.

RESULTS

From 2014 November to 2015 January, 38 samples with detectable viremia were collected to sequence the NS3 gene for the screening of the Q80K mutation in patients undergoing the therapy with SMV+ peginterferon and ribavirin. The median age of the patients was 48 years, and 32/38 of them were males. The median value of the viral load was about 900,000 UI/mL. The genotype sequences were similarly distributed between the clade 1 (53%) and the clade 2 (47%).

Two out of 38 samples in this study showed the

Table 1 - Susceptibility profile, supplied by Geno2pheno, on the 38 study samples.

Interpretation	*Protease NS3 inhibitors*					
	Simeprevir		*Telaprevir*		*Boceprevir*	
	No.	*%*	*No.*	*%*	*No.*	*%*
Susceptibility	36	94.7	22	57.9	37	97.4
Possible resistance	0	0	16	42,1	0	0
Resistance	2	5.3	0	0	1	2.6

Q80K mutation associated with pharmacoresistance to SMV. In 16 samples, mutations associated to possible pharmacoresistance to telaprevir, a linear protease inhibitor, were found. In one sample was detected the T54S mutation, which is responsible for resistance to boceprevir and is also a linear protease inhibitor (Table 1).
Both in patients showing the Q80K and in the subject with the T54S, the virus belonged to clade 1 of the genotype 1a. Twelve patients showed mutations not associated with pharmacoresistance, even though observed in sites of possible pharmacoresistance; among them, N174S was the most frequent (14/38, 37%), followed by S122G (12/38, 32%). Finally, a large number of natural polymorfisms has been observed. The whole population showed the variation at the position 153 of the leucine - present in the reference sequence NC_004102 - with isoleucine. Other polymorphisms at high frequency are: S91A (76%), T40A (45%) and P67S (45%) (Tables 2a and 2b).

CONCLUSION

This paper shows that the prevalence of the Q80K mutation in genotype 1a patients is 5%. This value has to be compared with the literature data. So far, some differences have been observed in the circulation percentage of the Q80K mutation within the European population, with respect to the American population. A recent analysis shows that in North America the Q80K polymorphism is present in 34% of the HCV infected population, and in 48% of patients with G1a. In Europe, the prevalence is lower: 6% of European patients show the Q80K mutation that is, moreover, present in 19% of the 1a genotypes.

Table 2a - Cheat sheet of the mutations in 38 patients under observation. Red: mutations associated with drug resistance; Yellow: mutations associated with possible drug resistance.

	Pz	I18V	Q28EQ	Q28E	Q28T	Q28G	V33I	T40A	Q41H	T54S	V55I	T61S	R62K	I64M	I64L	P67S	P67PS	Q80K	Q80LQ	V83I	P86S	A87S	S91A	S91T
1	CF											•											•	
2	GG	•																					•	
3	BC	•			•							•	•										•	
4	CG	•					•			•	•												•	
5	PG															•					•		•	
6	DM															•							•	
7	VA			•				•								•							•	
8	PI	•	•													•						•	•	
9	MA							•								•							•	
10	TN					•		•								•								
11	DC							•												•			•	
12	SV							•											•					
13	ZD							•								•							•	
14	MV															•							•	
15	BS						•									•								
16	BP							•								•							•	
17	BG								•				•	•							•		•	
18	BS																						•	
19	CP																						•	
20	DA							•									•						•	
21	MR							•							•			•						•
22	MV							•															•	
23	AR	•						•										•					•	
24	PV							•								•								
25	BG															•							•	
26	PP							•													•		•	
27	SL								•							•							•	
28	ER	•	•					•								•								•
29	NR							•								•								
30	EM																•						•	
31	LL								•				•										•	
32	DS			•																			•	
33	CA							•												•			•	
34	VB							•							•									
35	TF	•					•							•									•	
36	NV															•					•		•	
37	CG											•				•							•	
38	ZC				•							•										•		

Table 2b - Cheat sheet of the mutations in 38 patients under observation. Red: mutations associated with drug resistance; Yellow: mutations associated with possible drug resistance.

	Pz	T95IT	A95S	T98A	H110Q	I114V	R117C	R119Q	S122G	G124A	I132IV	S133FS	A147V	A147AV	L153I	I170V	N174S	N174G	E176Q
1	CF														•	•			
2	GG														•				
3	BC				•				•						•				
4	CG				•										•				•
5	PG				•										•				
6	DM						•		•						•		•		
7	VA	•													•				
8	PI								•						•		•		
9	MA								•	•			•		•				
10	TN								•						•			•	
11	DC											•			•		•		
12	SV													•	•				
13	ZD								•						•		•		
14	MV				•										•				
15	BS					•			•						•		•		
16	BP														•		•		
17	BG														•				
18	BS		•												•				
19	CP								•						•				
20	DA			•							•				•		•	•	
21	MR							•			•				•		•		
22	MV				•				•						•				
23	AR														•		•		
24	PV					•							•		•		•		
25	BG														•				
26	PP								•						•				
27	SL			•											•		•		
28	ER										•				•	•			
29	NR		•												•				
30	EM									•					•			•	
31	LL						•								•				
32	DS											•			•		•		
33	CA														•				
34	VB	•							•				•		•		•		•
35	TF				•						•				•				
36	NV													•	•			•	
37	CG							•	•						•				
38	ZC				•										•		•		

A more recent study reports 7.5% of Q80K mutation only in patients with genotype 1a; in particular, 0,5% of G1b patients (64% of G1 under analysis) show this mutation, whereas in G1a patients (36% of G1) 20% show the Q80K mutation [12]. The prevalence analyses described in this work - although executed on a low number of samples - could question the advices reported in the SMV technical data sheet, in line with the studies of Lenz et al. and of Sarrazin et al. [9, 10]. Their studies pointed out a Q80K percentage that is decidedly lower in Europe than that reported in American works. The different distribution of both clades of the genotype 1a and the prevalence of Q80K in the above clades could be responsible for this trend. In fact, the Q80K mutation detected in the subtypes 1a has been observed, so far, only in the sequences classified as clade 1. In Italy, the clade 1 represents 47% of G1a, while in North America 73% of the genotypes 1a belongs to the clade 1. Recent studies have highlighted the high toxicity of DAA in special patient populations; in particular, the SMV produces hepatotoxicity in patients with advanced cirrhosis [12, 14]. Therefore, it is necessary to conduct further epidemiological studies on the Q80K prevalence in Italy to evaluate the cost-to-benefit ratio of screening tests before starting the SMV therapy.

Conflict of interest: The authors declare that there aren't conflicts of interest of any kind or nature.

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