Annals of Gastroenterology (2019) **32,** 88-92

Intergenotypic 2k/1b hepatitis C virus recombinants in the East Macedonia and Thrace region of Greece

Katerina Kassela[a,b], Ioannis Karakasiliotis[b,c], Eleni Kokkiou[a], Fani Souvalidou[a], Panayotis Mimidis[a], Stavroula Veletza[c], Maria Panopoulou[d], John Koskinas[e], Konstantinos Mimidis[f], Penelope Mavromara[a,b]

Democritus University of Thrace, Alexandroupolis; Hellenic Pasteur Institute, Athens; Medical School of Athens, Hippokration Hospital Athens, Greece

Abstract

Background Intergenotypic recombinant hepatitis C virus (HCV) strains emerge rarely during coinfection of the same individual with two HCV genotypes. Few recombinant HCV strains have been identified to date and only one, CRF01 2k/1b, has become a worldwide concern. This study reevaluated the genotyping of three HCV genotype 2 strains from a group of patients with an unusually low rate of sustained virological response after pegylated interferon/ribavirin treatment. In addition, genetic determinants of host interferon resistance were evaluated.

Methods The HCV type 2 strains from the patients' serum were subjected to partial sequencing of the core-E1, NS2, NS5A and NS5B regions by reverse transcription polymerase chain reaction. Furthermore, the IFNL3 rs12979860 and the IFNL4 rs368234815 single nucleotide polymorphisms were defined in two of the three patients.

Results All three strains were phylogenetically related to the Russia-derived CRF01 2k/1b while they encompassed the exact same 2k/1b junction site within NS2.

Conclusion This is the first report of HCV 2k/1b recombinants in Greece and the greater area of the Balkans.

Keywords Hepatitis C virus, IFN-resistance, phylogenetic analysis, recombination

Ann Gastroenterol 2019; 32 (1): 88-92

[a]Department of Molecular Biology and Genetics, Democritus University of Thrace, Alexandroupolis (Katerina Kassela, Eleni Kokkiou, Fani Souvalidou, Panayotis Mimidis, Penelope Mavromara); [b]Laboratory of Molecular Virology, Hellenic Pasteur Institute, Athens (Katerina Kassela, Ioannis Karakasiliotis, Penelope Mavromara); [c]Laboratory of Biology, Department of Medicine, Democritus University of Thrace, Alexandroupolis (Ioannis Karakasiliotis, Stavroula Veletza); [d]Laboratory of Microbiology, Department of Medicine, Democritus University of Thrace, Alexandroupolis (Maria Panopoulou); [e]Department of Internal Medicine, Medical School of Athens, Hippokration Hospital Athens (John Koskinas); [f]1st Department of Internal Medicine, Democritus University of Thrace Medical School, Alexandroupolis (Konstantinos Mimidis), Greece

Conflict of Interest: None

Correspondence to: Ioannis Karakasiliotis, Laboratory of Biology, Department of Medicine, Democritus University of Thrace, University Campus Dragana, 68100 Alexandroupolis, Greece, e-mail: ioakarak@med.duth.gr

Received 5 March 2018; accepted 25 September 2018; published online 2 November 2018

DOI: https://doi.org/10.20524/aog.2018.0322

Introduction

Hepatitis C virus (HCV) infection presents a major global health burden. Chronic HCV infection may lead to severe clinical outcomes, such as cirrhosis and hepatocellular carcinoma [1]. HCV is a positive sense RNA virus belonging to the *Flaviviridae* family. The viral genome encodes for a polyprotein cleaved by host and viral proteases to produce the individual viral proteins. HCV is characterized by an extensive genetic variability that has an impact on disease progress and treatment outcome. Genotypes 1, 3 and 4 are characterized by a higher incidence of hepatocellular carcinoma and low rates of sustained virological response (SVR) following pegylated interferon (peg-IFN)/ribavirin therapy [2]. On the other hand, genotype 2 is more likely to be cleared spontaneously, while it shows more than 80% response to peg-IFN/ribavirin therapy [3]. The development of new, direct-acting antivirals (DAAs) against HCV has increased SVR rates to more than 95%; however, their high cost has limited their availability [4]. The first HCV recombinant to be identified was the CRF01 strain from St. Petersburg in Russia, comprising a rare 2k 5' moiety

followed by a 1b 3' moiety [5]. Since then, 2k/1b recombinant strains have been identified in countries bordering Russia and other European countries, including Ireland, France and Portugal [6-13]. In the present study we identified for the first time three 2k/1b recombinant clinical isolates in the Greek region of East Macedonia and Thrace.

Patients and methods

Patients

Patients who received peg-IFN/ribavirin treatment in the First Department of Internal Medicine of the University Hospital of Alexandroupolis, Thrace, Greece showed an unusually low rate of SVR. The study protocols were approved by the institutional ethics committee. Written informed consent was obtained from all the patients.

Patient 1 In October 2004, a 46-year-old male of Georgian decent with a history of alcohol abuse and intravenous drug use was referred to our hospital because of thrombocytopenia and increased transaminase levels. Laboratory results revealed thrombocytopenia, elevated transaminase levels (alanine aminotransferase [ALT] 89 IU/L, aspartate aminotransferase 117 IU/L) and γ-glutamyl transpeptidase 257 IU/L. There were no other abnormalities. Serological testing was negative for hepatitis B surface antigen, HIV, and hepatitis A virus. Serological testing for HCV was positive. HCV RNA was detected (viral load 6.78×10^5 IU/mL) and genotype 2a was identified by INNOLiPA. The patient received treatment with peg-IFN/ribavirin for 24 weeks but showed no SVR six months after the end of treatment. For the next 10 years we lost follow up of our patient until July 2014, when he was again referred to our hospital for decompensated cirrhosis with ascites. Endoscopy revealed small esophageal varices. A contrast-enhanced ultrasound and magnetic resonance imaging of the liver revealed a 35 mm mass in segment V of the right lobe. The patient refused any surgical intervention, including the possibility of liver transplantation. He was discharged with symptomatic treatment (spironolactone 25 mg/day, furosemide 40 mg/day).

Patient 2 In February 2010, a 60-year-old male from Georgia with a history of an untreated chronic HCV infection from June 2000 presented to our outpatient clinic because of increased ALT (186 IU/L) levels. HCV RNA was detected (1.7×10^6 IU/mL) and genotype 2a/2c was identified by INNOLiPA. A diagnostic liver biopsy revealed features of liver cirrhosis. The patient was offered a 24-week course of peg-IFN/ribavirin, but stopped treatment after 8 weeks because of side-effects (flu-like syndrome and depression). He refused any additional treatment and is now followed up every six months.

Patient 3 In January 2013, a 35-year-old male from Georgia, ex-drug user, visited our outpatient clinic because of a newly diagnosed HCV infection. His HCV viral load was 5.6×10^6 IU/mL and genotype 2a/2c was identified by INNOLiPA. A liver FibroScan revealed 4.3 kPa compatible with no fibrosis (F0). Treatment was initiated with peg-IFN/ribavirin, but at the end of treatment (24 weeks) viremia persisted (3.8×10^5 IU/mL). Two years after the initial treatment a second therapeutic course was given with the same drugs. This time viremia remained undetectable at the end and at follow up 6 months after, indicating SVR.

HCV genotyping and phylogenetic analysis

To identify a possible reason for the interferon resistance, we sequenced the core/E1 region of strains to reevaluate genotyping. Serum samples from the three HCV type 2 infected patients were used in this study. Serum from patients 1, 2 and 3 were obtained in 2014, 2015 and 2015, respectively. HCV RNA was extracted using the NucleoSpin Dx Virus kit (Macherey-Nagel) and reverse-transcribed to complementary DNA using M-MLV Reverse Transcriptase (Promega). Core_F and Core_R primers were used to amplify a 1028 bp 5' UTR-Core-E1 polymerase chain reaction (PCR) product, while NS2_F and NS2_R primers were used for amplification of the NS2 region. Amplification of NS5A (520 bp) and NS5B (450 bp) regions was performed using primer pairs NS5A_F/NS5A_R and NS5B_F/NS5B_R, respectively (Table 1). The PCR products were purified using NucleoSpin gel and PCR clean-up purification kit (Macherey-Nagel), and then the HCV sequences were determined by cycle sequencing. The sequencing reactions were carried out using a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with the automated capillary DNA Sequencer ABI 3730 XL Analyzer (Applied Biosystems), according to the manufacturer's instructions. GenBank accession numbers of the sequences obtained in this study are MH883229-MH883231 for the Core-E1 sequences, MH883226-MH883228 for the NS2 sequences and MH883232-MH883234 for the NS5A sequences.

The phylogenetic analysis was based on the core/E1 region of 2k/1b strains identified in Europe from patients originating from Azerbaijan (FJ435462, FJ435480, FJ435490, FJ435497, FJ435505, FJ435514), Georgia (EU684686, EU684728, JF949903, JF949904, JF949905, JF949906, JF949907, FJ821465), Russia (JF949908, AB327018, AY070214, AY070215, AB327015, AB327011, AB327010, AB327012, AY587845, AB327013, AB327014), and Uzbekistan (AB327016, AB327122). Evolutionary analysis in the Core/E1 and NS5A regions was conducted using MEGA7 [14] by applying the neighbor-joining method [15]. The evolutionary distances were computed using the Maximum Composite Likelihood method [16,17] and are in the units of the number of base substitutions per site. Genotyping of rs12979860 was performed by PCR using oligos IFNL3F and IFNL3R, resulting in the amplification of a 242 fragment and RFLP with restriction endonuclease BstU1. Genotyping of rs368234815 was performed by PCR using oligos IFNL4F and IFNL4R, resulting in the amplification of a 203 fragment and RFLP with restriction endonuclease Eco0109I (Table 1).

Results

Sequence analysis of the core-E1 region revealed that the three patients were infected with HCV strains phylogenetically

Table 1 Oligonucleotides used in the study

Primer name	Sequence	Genotype	Region/SNP	PCR product (bp)
CORE_F	5' GCCTGATAGGGTGCTTGCGAGTGCC 3'	2	5' UTR-CORE-E1	1028
CORE_R	5' CAATTCAGCATCATGTCCCATGCCAT 3'			
NS2_F	5' TAGTGTTTGACATAACCAAGTGGC 3'	2k	NS2	370
NS2_R	5' CCCCTTCGGGCGGAGACG 3'	1b		
NS5A_F	5' AGCTCCCATGYGAGCCCGA 3'	1b	NS5A	520
NS5A_R	5' CTCCGTGGAGGYGGTATYGGA 3'			
NS5B_F	5' TATGAYACCCGMTGYTTYGAC 3'	1,2,3	NS5B	450
NS5B_R	5' GAGGARCAKGATGTTATYAGCTC 3'			
IFNL3F	5' GCTTATCGCATACGGCTAGG 3'		rs12979860	242
IFNL3R	5' AGGCTCAGGGTCAATCACAG 3'			
IFNL4F	5' ACTTACGTAGCGGTCCCTCA 3'		rs368234815	203
IFNL4R	5' CTCTCTTTGGCTTCCCTGAC 3'			

PCR, polymerase chain reaction, SNP, single nucleotide polymorphism

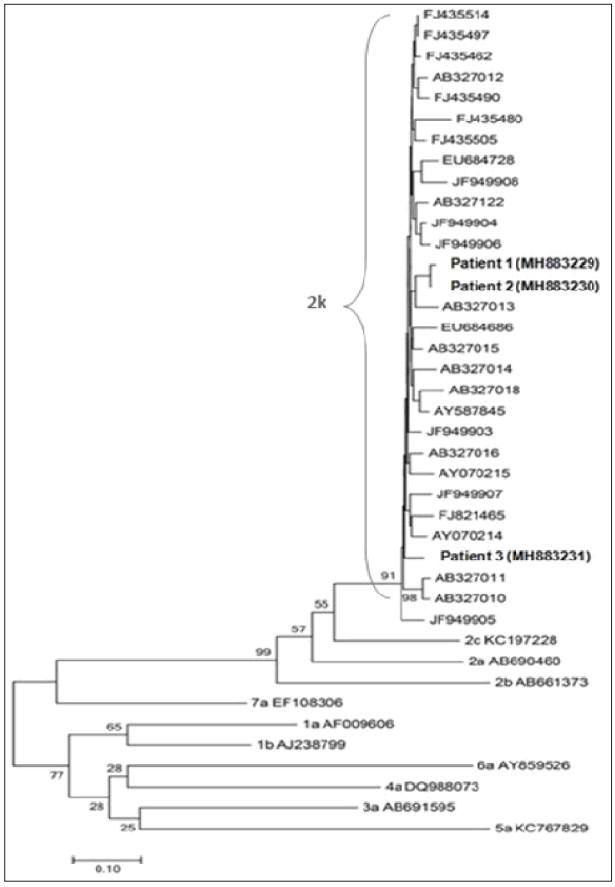

Figure 1 Evolutionary relationships of taxa in the core/E1 region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [21]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 40 nucleotide sequences of the core/E1 region. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 263 positions in the final dataset

related to the Russia-derived CRF01 2k/1b strain. The core/E1 region of all three strains, originally genotyped as 2a or 2a/2c, clustered together with the 2k core/E1 region of the 2k/1b intergenotypic recombinants (Fig. 1). Following partial sequencing of the NS5A and NS5B regions we verified the HCV 1b origin of the regions in all three patients (Fig. 2). Sequencing analysis of the NS2 region, where the recombination was anticipated, revealed that all three strains encompassed the same recombination site as the original RF1 2k/1b strain from Russia at genomic position 3175 (Fig. 3) [10]. The identification of the particular recombination site established the RF1 2k/1b origin of the strains as every recombinant HCV strain identified up to date has a unique recombination site position.

In parallel with the reassessment of virus genotype, we performed analysis of the genetic background of the patients as it has been linked to the treatment outcome. Thus, we identified the IFNL3 rs12979860 and the IFNL4 rs368234815 genotypes/variants of these patients. Patient 1 was genotyped as ΔG/ΔG for rs368234815 and TT for rs12979860, both favoring a poor outcome of treatment. Patient 3 was genotyped as TT/ΔG for rs368234815 and CC for rs12979860, favoring poor outcome and good outcome respectively.

Discussion

Intergenotypic recombinant HCV strains emerge rarely during coinfection of the same individual with two HCV genotypes. For reasons that have not been adequately addressed, the 5' moiety is derived mostly from HCV genotype 2, while the 3' part varies [18]. All HCV intergenotypic recombinants identified to date are restricted to a single individual [18]. The only strain that has been shown to circulate is the RF1 2k/1b strain, which was initially identified in Russia and other neighboring countries [5,7,10,19]. However, during the last decade a number of publications have shown

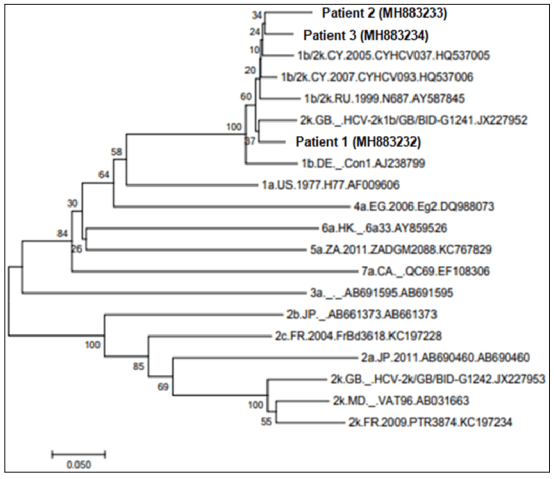

Figure 2 Evolutionary relationships of taxa in the NS5A region. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [21]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 20 nucleotide sequences of the NS5A region. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 404 positions in the final dataset

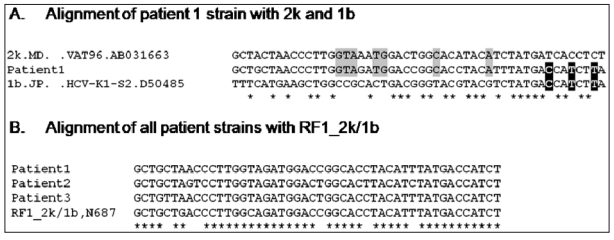

Figure 3 (A) Alignment of Patient 1 NS2 region recombination site with sequences of 2k and 1b origin (Grey = similarity with 2k, black = similarity with 1b). (B) Alignment of NS2 recombination site of all three patients with RF1_2k/1b strain

the spread of RF1 2k/1b in other European countries, from individuals of Russian or Georgian origin [6-10]. This is the first study to report the circulation of recombinant 2k/1b strains in Greece or the Balkan Peninsula. All patients had lived in Georgia, suggesting an imported infection. The ability of this recombinant strain to spread in the population, especially among intravenous drug users (IDU) underlines the high possibility of the virus spreading into the indigenous population.

There are contradicting indications regarding the response of RF1 strain to IFN therapy. A recent report on 2k/1b strains from Georgia showed lower ratios of SVR (<50%) to pure HCV 1b genotype, possibly due to the 24-week rather than 48-week regimen followed in genotype 1b patients [7]. Besides, the study of Susser *et al* suggested that genotype 1-based DAAs are very effective in eradication of 2k/1b virus, compared to the standard sofosbuvir plus ribavirin treatment for HCV genotype 2 [20]. Our results indicate that 2k/1b strains show mild resistance to IFN treatment, as both patients 1 and 3 failed to develop SVR after IFN administration. The latter patient managed to develop SVR on his second treatment, which may be associated with the favorable IFNL3 rs12979860 CC background. On the other hand, patient 1 had a genetic background favoring a poor outcome of treatment, according to IFNL3 rs12979860 and IFNL4 rs368234815 genotypes/variants. Therefore, the coexistence of a nonstructural region of 1b genotype, which is more resistant to IFN than genotype 2, and a non-favorable SNP would rather suggest a cumulative effect on treatment outcome. Patient 2, who stopped treatment after 8 weeks, has now agreed to receive DAAs. Thus, given the wide distribution of RF1 2k/1b in certain areas, it is important to check all HCV genotype 2 isolates for the presence of recombination.

In summary, this is the first identification of 2k/1b recombinants in Greece, possibly imported from Georgia. Further analysis of the spread of the strain in the indigenous population of the area of East Macedonia and Thrace, where all three current incidences were reported, will be required, especially within the IDU community.

Summary Box

What is already known:

- Numerous recombinant hepatitis C virus (HCV) strains have been identified to date
- Only one HCV strain, CRF01 2k/1b, from Russia, has become a worldwide concern
- There are contradictory indications regarding the response of CRF01 strain to interferon (IFN) therapy

What the new findings are:

- Three HCV strains, phylogenetically related to the Russia-derived CRF01 2k/1b, were identified
- This is the first identification of 2k/1b recombinants in Greece
- 2k/1b strains showed mild resistance to IFN treatment

References

1. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003;**362**:2095-2100.
2. Simmonds P. Genetic diversity and evolution of hepatitis C virus—15 years on. *J Gen Virol* 2004;**85**:3173-3188.
3. Echeverría N, Moratorio G, Cristina J, Moreno P. Hepatitis C virus genetic variability and evolution. *World J Hepatol* 2015;**7**:831-845.
4. Zhang J, Nguyen D, Hu KQ. Chronic hepatitis C virus infection: a review of current direct-acting antiviral treatment strategies. *Am J Med Sci (Boston)* 2016;**9**:47-54.
5. Kalinina O, Norder H, Mukomolov S, Magnius LO. A natural

intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002;**76**:4034-4043.
6. Demetriou VL, Kyriakou E, Kostrikis LG. Near-full genome characterisation of two natural intergenotypic 2k/1b recombinant hepatitis C virus isolates. *Adv Virol* 2011;**2011**:710438.
7. Karchava M, Waldenstrom J, Parker M, et al. High incidence of the hepatitis C virus recombinant 2k/1b in Georgia: Recommendations for testing and treatment. *Hepatol Res* 2015;**45**:1292-1298.
8. Moreau I, Hegarty S, Levis J, et al. Serendipitous identification of natural intergenotypic recombinants of hepatitis C in Ireland. *Virol J* 2006;**3**:95.
9. Morel V, Descamps V, François C, et al. Emergence of a genomic variant of the recombinant 2k/1b strain during a mixed hepatitis C infection: a case report. *J Clin Virol* 2010;**47**:382-386.
10. Raghwani J, Thomas XV, Koekkoek SM, et al. Origin and evolution of the unique hepatitis C virus circulating recombinant form 2k/1b. *J Virol* 2012;**86**:2212-2220.
11. Ramiere C, Tremeaux P, Caporossi A, et al. Recent evidence of underestimated circulation of hepatitis C virus intergenotypic recombinant strain RF2k/1b in the Rhone-Alpes region, France, January to August 2014: implications for antiviral treatment. *Euro Surveill* 2014;**19**.
12. Stelzl E, Haas B, Bauer B, et al. First identification of a recombinant form of hepatitis C virus in Austrian patients by full-genome next generation sequencing. *PLoS One* 2017;**12**:e0181273.
13. Avó AP, Agua-Doce I, Andrade A, Pádua E. Hepatitis C virus subtyping based on sequencing of the C/E1 and NS5B genomic regions in comparison to a commercially available line probe assay. *J Med Virol* 2013;**85**:815-822.
14. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016;**33**:1870-1874.
15. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;**4**:406-425.
16. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN (editor): Mammalian protein metabolism. Academic Press, New York; 1969, pp. 21-132.
17. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A* 2004;**101**:11030-11035.
18. González-Candelas F, López-Labrador FX, Bracho MA. Recombination in hepatitis C virus. *Viruses* 2011;**3**:2006-2024.
19. Zakalashvili M, Zarkua J, Weizenegger M, et al. Identification of hepatitis C virus 2k/1b intergenotypic recombinants in Georgia. *Liver Int* 2018;**38**:451-457.
20. Susser S, Dietz J, Schlevogt B, et al. Origin, prevalence and response to therapy of hepatitis C virus genotype 2k/1b chimeras. *J Hepatol* 2017;**67**:680-686.
21. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;**39**:783-791.