MOLECULAR EPIDEMIOLOGY OF NON-1B HCV STRAINS INFECTING ROMANIAN PATIENTS

Sorin Dinu[1,2*], Grațiela Țârdei[3], Cristina Calomfirescu[3], Adriana Moțoc[3], Augustina Maria Culinescu[3], Simin Aysel Florescu[3], Camelia Sultana[4,5], Simona Ruță[4,5], Emanoil Ceauşu[3], Gabriela Oprişan[1,6]

[1]Cantacuzino National Medico-Military Institute for Research and Development, Molecular Epidemiology Laboratory, Bucharest, Romania; [2]The Research Institute of the University of Bucharest (ICUB), Earth, Environmental and Life Sciences Division, Bucharest, Romania; [3]Dr Victor Babeş Clinical Hospital for Infectious and Tropical Diseases, Bucharest, Romania; [4]Carol Davila University of Medicine and Pharmacy, Bucharest, Romania; [5]Ştefan S. Nicolau Institute of Virology, Bucharest, Romania; [6]Faculty of Pharmacy, Titu Maiorescu University, Bucharest, Romania

ABSTRACT

Introduction: Through its severe complications, hepatitis C virus (HCV) infection is an important cause of liver disease worldwide. The numerous HCV genotypes and subtypes are widely spread, associated with different regions or populations/social groups, and display distinct response to antivirals. Some genotypes/subtypes are prone to mutation, making them less susceptible even to the direct-acting antivirals. HCV prevalence in Romania is considered the highest in Europe, predominating subtype 1b. Circulation of other HCV subtypes in Romania is documented.

Objectives: We conducted a study to characterize non-1b HCV isolates circulating in Romania between 2013 and 2014 in naïve patients.

Methods: Blood samples were used for HCV genotyping, phylogenetic analysis, detection of antiviral resistance mutations, and IL28B genotyping.

Results: Subtypes 1a, 3a and 4a were detected by sequencing a fragment of core region. Phylogenetic analysis showed high resemblance between the non-1b strains identified in this study and older Romanian strains. As well as the previous non-1b Romanian strains, most of the isolates in our study originated from intravenous drug users. The low genetic diversity of non-1b isolates circulating in Romania, corroborated with the epidemiological data, suggested that the number of introduction events of non-1b isolates in our country was limited and that they have been endemically circulating in a specific group. Subtype 1a strains were assigned to clade II, known to gather isolates not harboring NS3 Q80K polymorphism responsible for simeprevir resistance.

Conclusion: The transmission of non-1b HCV strains identified in this study could be related to the administration of intravenous drugs.

Keywords: hepatitis C virus, non-1b subtypes, genotyping, molecular characterization, intravenous drug users, clade II 1a isolates.

REZUMAT

Introducere: Prin complicațiile ei severe, infecția cu virusul hepatitei C (HCV) reprezintă o cauză importantă a bolii hepatice, la nivel global. Numeroasele genotipuri/subtipuri HCV sunt larg răspândite, au distribuție cunoscută, sunt asociate cu anumite populații/grupuri sociale și manifestă răspuns diferit la antivirale. Unele genotipuri/subtipuri sunt predispuse la mutații, fiind mai puțin susceptibile chiar și la noile antivirale cu acțiune directă. Prevalența HCV în România este considerată cea mai mare din Europa, predominând subtipul 1b. Circulația altor subtipuri în România este documentată.

Obiective: Am desfășurat un studiu pentru caracterizarea izolatelor non-1b circulante în România, între 2013-2014, la pacienți neexpuși la antivirale.

Metode: Au fost utilizate probe de sânge pentru genotiparea HCV, analiza filogenetică, detecția mutațiilor de rezistență la antivirale și genotiparea IL28B.

Rezultate: Subtipurile 1a, 3a și 4a au fost detectate prin secvențierea unui fragment din regiunea core. Analiza filogenetică arată asemănarea înaltă între tulpinile non-1b studiate și tulpini mai vechi din România.

**Corresponding author:* Sorin Dinu, Cantacuzino National Medico-Military Institute for Research and Development, Molecular Epidemiology Laboratory, 103 Splaiul Independenței, 050096, Bucharest, Romania, e-mail: sorind@cantacuzino.ro, phone: +40213069223

Ca și tulpinile anterioare, cele mai multe izolate din acest studiu provin de la pacienți care au declarat consumul de droguri injectabile. Diversitatea genetică scăzută a izolatelor non-1b circulante în România, coroborată cu datele epidemiologice, sugerează existența unui număr limitat de introduceri ale tulpinilor non-1b în țara noastră, precum și circulația endemică a acestora într-un anumit grup social. Tulpinile din subtipul 1a aparțin cladei II, cunoscută ca reunind izolate ce nu poartă mutația Q80K în proteina NS3, un polimorfism responsabil pentru rezistența la simeprevir.

Concluzie: Transmiterea tulpinilor HCV non-1b descrise în acest studiu poate fi legată de administrarea intravenoasă a drogurilor.

Cuvinte-cheie: virusul hepatitei C, subtipuri non-1b, genotipare, caracterizare moleculară, consumatori de droguri intravenoase, izolate 1a din clada II.

INTRODUCTION

Hepatitis C virus (HCV), the type member of *Flaviviridae* family (*Hepacivirus* genus, *Hepatitis C virus* species), is a small, enveloped, positive sense single-stranded RNA virus [1-3]. HCV isolates worldwide are highly diverse and currently classified into seven genotypes and sixty-seven subtypes with significant variations in the geographic distribution [4, 5]. Furthermore, at intra-host level the virus circulates as a complex mixture of quasispecies, representing the selection pool for different phenotypes [6]. Genotype 1 (G1) is the most prevalent globally (46%), followed by G3 (22%), G2 (13%), and G4 (13%). Subtype 1b accounts for 22% of all infections worldwide [5]. As reviewed elsewhere [7], the estimated prevalence varies from 0.5-0.7% in the Western Pacific Region and Americas to 2.3% in the Eastern Mediterranean Region, some countries such as Egypt exceeding regional prevalence.

Estimated prevalence for Romania (2.5%) is the highest in Europe [7], predominating subtype 1b isolates with more than 90% [8, 9]. However, subtypes 1a (5.4%), 4a (1.2%) and 3a (0.8%) have been also recorded in Romanian population and have been found in 54.8% of HCV patients who inject drugs [8, 10]. Molecular clock-based study performed on HIV-HCV co-infected patients suggests that non-1b strains emerged later in Romanian population [11].

We aimed to molecularly characterize non-1b HCV strains circulating in Romania between 2013 and 2014 by analyzing a sera collection established during a national grant concerning the study of HCV.

MATERIALS AND METHODS

Patients and samples

Serum samples were collected from thirteen patients naïve to any antiviral treatment, prior to being subjected to interferon-ribavirin therapy. Viral load was evaluated at 4, 12 and 24 weeks of treatment to monitor the efficacy of treatment (*i.e.* EVR – early virological response; RVR – rapid virological response; SVR – sustained virological response). IL28B genotype was determined in order to predict the outcome of the interferon-ribavirin therapy. Patients were enrolled between 2013 and 2014 in the framework of a national grant – HepGen 88/2012 – concerning HCV in Romania. Study was approved by the Bioethics Committee of Cantacuzino Institute.

RNA extraction and reverse transcription

Viral RNA was extracted from 140 μL serum using QIAamp Viral RNA Mini Kit (Qiagen, Hilden Germany) according to the kit's insert. Reverse transcription was carried out as previously described [12].

Genotyping and phylogenetic analysis

A 422 bp fragment in the core region was amplified using a semi-nested PCR as previously described [8]. PCR products were gel-purified using a commercial kit (Wizard® SV PCR and Gel Clean-Up System, Promega, WI, USA) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer (Applied Biosystems, CA, USA). Raw sequences were visually inspected and proofed with BioEdit version 7.2.5 [13]. Genotype/subtype was assessed by using NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) and by enquiring The Los Alamos hepatitis C sequence database

[14]. The phylogenetic analysis was conducted using Mega 7 software [15].

Detection of antiviral resistance mutations in NS3 and NS5A genomic regions

Fragments of NS3 and NS5A genomic regions were PCR amplified and sequenced using primers described elsewhere [16]. Resistance mutations were detected with Geno2pheno [hcv 0.92] online tool [17], which was also used to assign the clade for subtype 1a isolates.

IL28B (rs12979860) genotyping

Polymorphism rs12979860 near the IL28B human gene was assessed as previously described [18].

RESULTS

From the total number of samples genotyped (n=158) during the HepGen 88/2012 project (unpublished data), the distribution of HCV subtypes was as follows: 1b – 145 samples (91.77%), 1a – eight samples (5.06%), 3a – three samples (1.9%) and 4a – two samples (1.27%). Non-1b samples were further analyzed in the present study. Female/male ratio in non-1b group was 0.3, majority of the cases were from urban area (n=11), with a median age of 29 years. All patients declared at least one risk factor associated with HCV transmission (Table 1). SVR was achieved in five cases. The status at the end of the treatment was not assessed for the rest of the patients. Since the number of studied patients is very limited, no conclusion regarding the association between the outcome of the treatment and Il28B genotype can be drawn. However, unfavorable genotypes (CT and TT) were observed in the cases of patients achieving SVR (Table 1).

Phylogenetic analysis was performed on a 269 nt fragment spanning positions 390-658 in H77 genome (GenBank acc. no. AF009606). Isolates analyzed in this study are closely related to isolates from 2004, 2006 and 2008 identified in Romanian patients. Some of the similar sequences were obtained from intravenous drug users (Fig. 1). Ten out of the thirteen patients enrolled in our study declared a history of intravenous drug use.

No resistance mutations were detected in NS5A region. A single patient harbored V36L substitution in NS3 region, known to confer resistance to boceprevir and telaprevir – first generation direct-acting antivirals. NS3 based-typing assigned all 1a isolates into clade II.

DISCUSSION

With approximately 71 million chronically infected persons, HCV is an important cause of morbidity and mortality across the globe due to its severe complications [19]. The classical interferon-ribavirin regimen has an unsatisfactory success rate of 40-50% [20]. Success rate is influenced by viral genotype and mutations, different clinical parameters, and host IL28B polymorphisms [21-24].

In the present study we have analyzed samples from Romanian chronic hepatitis C patients infected with non-1b isolates. The estimated HCV prevalence in Romania is considered to be the highest in Europe [7] with significant differences between geographic regions and rural and urban areas [25]. Previous studies have shown the overwhelming prevalence of 1b isolates circulating in Romania – 86.4-99.6%, depending on the genotyping method used [8, 9, 26], and the modest rate of therapeutic success (38.9%) obtained after interferon-ribavirin treatment in the cases of 1b-infected Romanian patients [18]. Prevalence of other subtypes has been previously estimated, as follows: 1a (5.4%), 4a (1.2%) and 3a (0.8%) [8]. Consistent with past findings, our study showed similar prevalence of subtype 1a, 3a and 4a isolates in Romanian population. Non-1b isolates from previous studies have been associated with younger patients and intravenous drug users [8, 10, 27]. Genotyping study performed on HCV infected persons who inject drugs from Romania have shown an increased prevalence of non-1b genotypes, comparing to general population: 1a – 24%, 3a – 14.4%, and 4a – 7.7% [10]. Indeed, the patients from our study are young males and the majority of them have declared a history of intravenous drug use. Subtype 1a and genotypes 3 and 4 were also found more frequently in younger patients, males and intravenous drug users in a study from Spain, also [28]. The same study found genotype 3

Table 1. Demographic, virological and genetic characteristics of the studied patients and their clinical evolution during and after the interferon-ribavirin therapy

Patient	Age (years)	Sex (F/M)	Residence (urban/ rural)	History of intravenous drug use (yes/no)	Other risk factors associated with HCV transmission (yes/no)	Viral subtype	IL28B genotype	RVR[1] (yes/no)	EVR[2] (yes/ no)	SVR[3] (yes/ no)
SVB-022	24	M	U	yes	yes	4a	TT	no	n/a	n/a
SVB-067	22	F	R	yes	yes	1a	TT	yes	n/a	yes
SVB-074	31	M	U	yes	yes	1a	CT	yes	n/a	yes
SVB-087	24	F	U	yes	yes	1a	TT	yes	n/a	yes
SVB-092	22	M	U	yes	yes	1a	CT	yes	n/a	yes
SVB-093	26	M	U	no	yes	1a	CT	yes	yes	n/a
SVB-095	29	M	U	no	yes	4a	TT	no	yes	n/a
SVB-096	30	F	U	no	yes	1a	CC	yes	n/a	n/a
SVB-098	33	M	U	yes	yes	3a	CT	no	yes	n/a
SVB-099	35	M	U	yes	yes	1a	CT	no	no	yes
SVB-104	30	M	U	yes	yes	3a	CC	no	yes	n/a
SVB-112	24	M	U	yes	yes	3a	n/a	no	no	n/a
SVB-129	35	M	R	yes	yes	1a	n/a	no	n/a	n/a

[1]RVR – rapid virological response (HCV RNA not detectable after 4 weeks of treatment); [2]EVR – early virological response (HCV RNA not detectable after 12 weeks of treatment); [3]SVR – sustained virological response (HCV RNA not detectable at 24 weeks – *i.e.* completion of treatment)

more frequent in Asian and Eastern European immigrants and subtype 1a and genotypes 3 and 4 to be linked to HIV co-infection. Phylogenetic analysis showed the high degree of relatedness between isolates from this study and isolates infecting Romanian patients between 2004 and 2008. Some of these sequences originated also from intravenous drug users.

The low level of genetic diversity among non-1b isolates from Romania, corroborated with the information collected from patients, indicates that these strains share a common origin and they have been transmitted via the same route in a distinct social group. Recent study performed on Romanian HIV-HCV co-infected patients concluded that non-1b HCV strains from these cases originated later in Romanian population [11].

Phylogenetic studies have shown that isolates belonging to subtype 1a are segregated into two clades [29]. Clade I showed an earlier origin from the common ancestor compared with clade II. Clade I isolates are more prevalent in non-European countries, represented mostly by the United States. The European isolates are almost equally distributed in the two clades [30]. All 1a isolates in this study fall into clade II.

Direct-acting antivirals (DAAs) have raised the chances of obtaining viral clearance in more than 95% of chronic hepatitis C patients [31] but genotype/subtype-specific re-

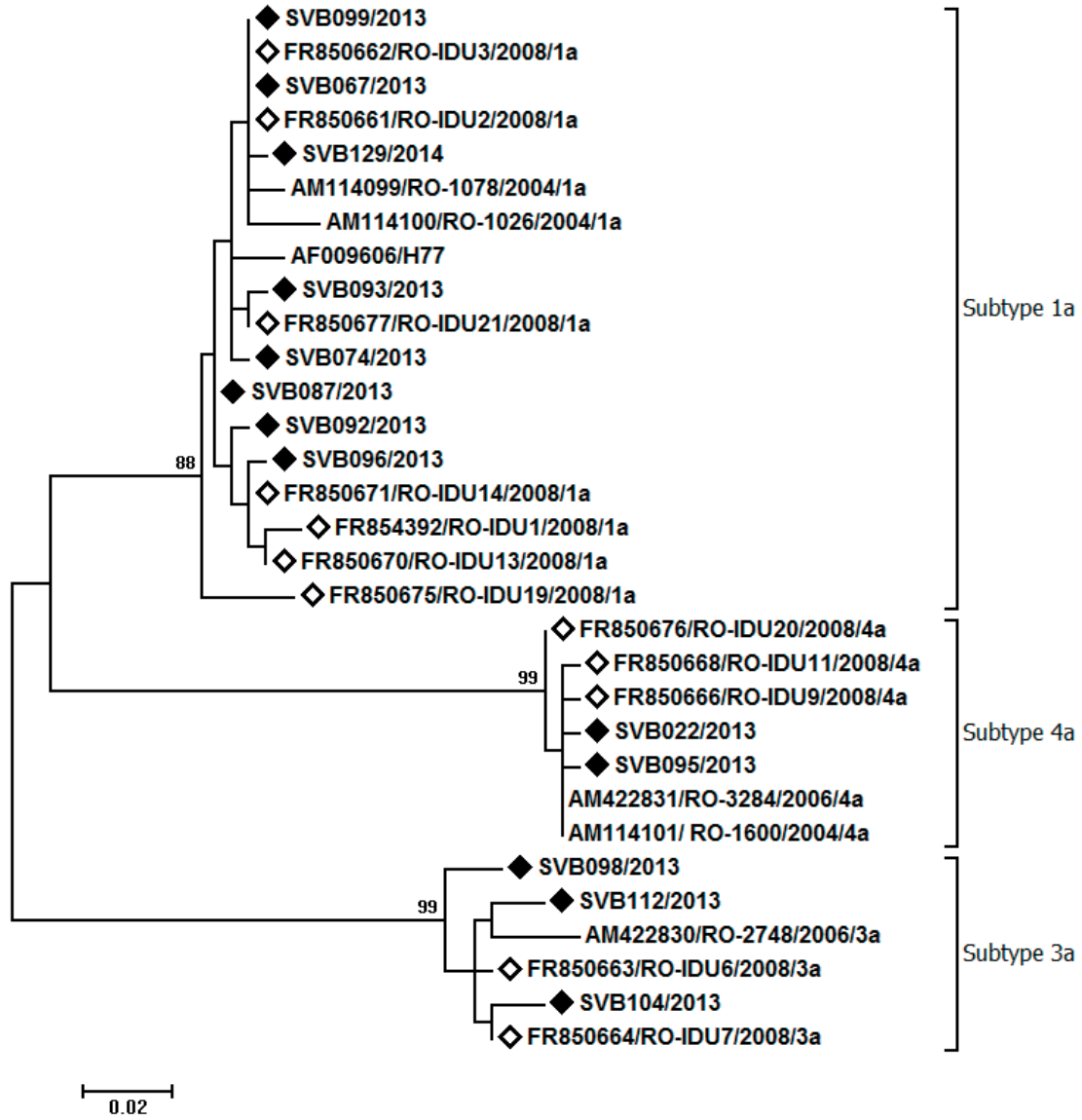

Fig. 1. Maximum-likelihood analysis depicting relatedness of the studied HCV isolates to other non-1b strains originating from Romanian patients. Black diamond: sequence from this study; white diamond: sequence from other study and originating from intravenous drug user.
Dendrogram was constructed with Mega 7 software [15], Kimura-2 parameter, 1000 bootstrap replications. Values at nodes represent bootstrap percentages (values < 70% are not shown).

sistance mutations, with different prevalence are widely distributed [32]. Therefore, viral genotyping and, in some cases, detection of resistance mutations should be performed before establishing an optimal antiviral regimen [33]. It has been shown that prevalence of resistance mutations in subtype 1a isolates is greater than in subtype 1b [32] and testing for the presence of NS3 Q80K polymorphism is recommended for 1a isolates before starting a simeprevir based regimen as well as searching for resistant variants at positions 155, 156 and 168 in NS3 region of genotype 1 isolates [34]. Our resistance mutation analysis only found NS3 V36L substitution in a single patient. This mutation, detected in naïve patients from other studies, is known to confer low level of resistance to triple therapy and does not affect the viral fitness [35, 36]. Moreover it has been shown that polymorphism NS3 Q80K is not found in clade II

1a isolates [30], and, fortunately, this is also the case of the samples analyzed here.

Characterization of HCV isolates is not only of epidemiological importance, but is critical for the clinical evolution of the patient, specially when selecting a therapeutic approach. Although a pan-genotype treatment displaying high genetic barrier to resistance is desirable, the success of currently available therapeutic alternatives is still influenced by the subtype/genotype of the infecting isolate, and sometimes by natural polymorphisms harbored by it.

CONCLUSIONS

Although limited, our study shows the circulation of non-1b HCV strains among young patients with a history of intravenous drug use.

Acknowledgements

This work was partially supported by a grant of the Romanian National Authority for Scientific Research, CNDI–UEFISCDI, project number 88/2011.

Conflict of interests: None to declare.

REFERENCES

1. Fields BN, Knipe DM, Howley PM. Fields Virology. 5th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007.
2. Smith DB, Becher P, Bukh J, Gould EA, Meyers G, Monath T, et al. Proposed update to the taxonomy of the genera Hepacivirus and Pegivirus within the Flaviviridae family. J Gen Virol. 2016;97(11):2894-907.
3. Simmonds P, Becher P, Bukh J, Gould EA, Meyers G, Monath T, et al. ICTV Virus Taxonomy Profile: Flaviviridae. J Gen Virol. 2017;98(1):2-3.
4. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology. 2014;59(1): 318-27.
5. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. J Hepatol. 2014;61(1 Suppl):S45-57.
6. Echeverria N, Moratorio G, Cristina J, Moreno P. Hepatitis C virus genetic variability and evolution. World J Hepatol. 2015;7(6):831-45.
7. Calvaruso V, Petta S, Craxi A. Is global elimination of HCV realistic? Liver Int. 2018;38(Suppl 1):40-6.
8. Sultana C, Oprisan G, Szmal C, Vagu C, Temereanca A, Dinu S, et al. Molecular epidemiology of hepatitis C virus strains from Romania. J Gastrointestin Liver Dis. 2011;20(3):261-6.
9. Manuc M, Preda CM, Popescu CP, Baicus C, Voiosu T, Pop CS, et al. New Epidemiologic Data Regarding Hepatitis C Virus Infection in Romania. J Gastrointestin Liver Dis. 2017;26(4):381-6.
10. Ruta S, Sultana C, Oprea C, Vagu C, Ceausu E, Cernescu C. HCV non-1b genotypes in injecting drug users from Romania. J Infect Dev Ctries. 2016;10(5):523-7.
11. Paraschiv S, Banica L, Nicolae I, Niculescu I, Abagiu A, Jipa R, et al. Epidemic dispersion of HIV and HCV in a population of co-infected Romanian injecting drug users. PLoS One. 2017;12(10):e0185866.
12. Dinu S, Calistru PI, Ceausu E, Tardei G, Oprisan G. Screening of Protease Inhibitors Resistance Mutations in Hepatitis C Virus Isolates Infecting Romanian Patients Unexposed to Triple Therapy. Roum Arch Microbiol Immunol. 2015;74(1-2):7-17.
13. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 1999;41:95-8.
14. Kuiken C, Yusim K, Boykin L, Richardson R. The Los Alamos hepatitis C sequence database. Bioinformatics. 2005. 21(3):379-84.
15. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016;33(7):1870-4.
16. Di Maio VC, Cento V, Lenci I, Aragri M, Rossi P, Barbaliscia S, et al. Multiclass HCV resistance to direct-acting antiviral failure in real-life patients advocates for tailored second-line therapies. Liver Int. 2017;37(4):514-28.
17. Kalaghatgi P, Sikorski AM, Knops E, Rupp D, Sierra S, Heger E, et al. Geno2pheno[HCV] - A Web-based Interpretation System to Support Hepatitis C Treatment Decisions in the Era of Direct-Acting Antiviral Agents. PLoS One. 2016;11(5):e0155869.
18. Sultana C, Oprisan G, Teleman MD, Dinu S, Oprea C, Voiculescu M, et al. Impact of hepatitis

C virus core mutations on the response to interferon-based treatment in chronic hepatitis C. World J Gastroenterol. 2016;22(37):8406-13.
19. Chen SL, Morgan TR. The natural history of hepatitis C virus (HCV) infection. Int J Med Sci. 2006;3(2):47-52.
20. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. Gut. 2006;55(9):1350-9.
21. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol. 2011;54(4):716-22.
22. Navaneethan U, Kemmer N, Neff GW. Predicting the probable outcome of treatment in HCV patients. Therap Adv Gastroenterol. 2009;2(5):287-302.
23. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;46(3):403-10.
24. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology. 2005;48(6): 372-80.
25. Gheorghe L, Csiki IE, Iacob S, Gheorghe C, Smira G, Regep L. The prevalence and risk factors of hepatitis C virus infection in adult population in Romania: a nationwide survey 2006 - 2008. J Gastrointestin Liver Dis. 2010;19(4): 373-9.
26. Oprisan G, Szmal C, Dinu S, Oprisoreanu AM, Thiers V, Panait M, et al. Comparative methods for genotyping hepatitis C virus isolates from Romania. Roum Arch Microbiol Immunol. 2009;68(3):151-7.
27. Sultana C, Vagu C, Temereanca A, Grancea C, Slobozeanu J, Ruta S. Hepatitis C Virus Genotypes in Injecting Drug Users from Romania. Cent Eur J Med. 2011;6(5): 672-8.
28. Acero Fernandez D, Ferri Iglesias MJ, Buxo Pujolras M, Lopez Nunez C, Serra Matamala I, Queralt Moles X, et al. Changes in the epidemiology and distribution of the hepatitis C virus genotypes in North-Eastern Spain over the last 35 years. Gastroenterol Hepatol. 2018;41(1):2-11.
29. Pickett BE, Striker R, Lefkowitz EJ. Evidence for separation of HCV subtype 1a into two distinct clades. J Viral Hepat. 2011;18(9):608-18.
30. De Luca A, Di Giambenedetto S, Lo Presti A, Sierra S, Prosperi M, Cella E, et al. Two Distinct Hepatitis C Virus Genotype 1a Clades Have Different Geographical Distribution and Association With Natural Resistance to NS3 Protease Inhibitors. Open Forum Infect Dis. 2015;2(2):ofv043.
31. Asselah T, Marcellin P, Schinazi RF. Treatment of hepatitis C virus infection with direct-acting antiviral agents: 100% cure? Liver Int. 2018;38(Suppl 1):7-13.
32. Chen ZW, Li H, Ren H, Hu P. Global prevalence of pre-existing HCV variants resistant to direct-acting antiviral agents (DAAs): mining the GenBank HCV genome data. Sci Rep. 2016;6:20310.
33. Sarrazin C. The importance of resistance to direct antiviral drugs in HCV infection in clinical practice. J Hepatol. 2016;64(2):486-504.
34. Wyles DL. Resistance to DAAs: When to Look and When It Matters. Curr HIV/AIDS Rep. 2017;14(6):229-37.
35. Shepherd SJ, Abdelrahman T, MacLean AR, Thomson EC, Aitken C, Gunson RN. Prevalence of HCV NS3 pre-treatment resistance associated amino acid variants within a Scottish cohort. J Clin Virol. 2015;65:50-3.
36. Bartels DJ, Sullivan JC, Zhang EZ, Tigges AM, Dorrian JL, De Meyer S, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naive patients prior to treatment. J Virol. 2013;87(3):1544-53.