Antiviral Research 95 (2012) 72–81

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Longitudinal analysis of the 5′UTR, E2-PePHD and NS5A-PKRBD genomic regions of hepatitis C virus genotype 1a in association with the response to peginterferon and ribavirin therapy in HIV-coinfected patients

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ARTICLE INFO

Article history:
Received 25 April 2012
Revised 22 May 2012
Accepted 26 May 2012
Available online 7 June 2012

Keywords:
HCV
Genotype 1a
HIV
Pegylated interferon
Ribavirin

ABSTRACT

Background: The rate of non-response to pegylated interferon plus ribavirin (peg-IFN + RBV) in HCV/HIV coinfected patients is higher than in HCV-monoinfected patients. In this sense, the contribution of HCV genetic variability is unknown. The 5′ untranslated (5′UTR), the nonstructural 5A (NS5A) and the second envelope (E2) HCV genomic regions have been implicated to peg-IFN therapy response. The proteins appear to block interferon (IFN)-induced RNA-dependent protein kinase (PKR) and the 5′UTR may influence the viral lymphotropism.

Methods: We examined comparatively the pretreatment HCV variability between HIV coinfected and HCV monoinfected patients as well as assessed longitudinally the impact of peg-IFN + RBV on HCV variability when HIV is co-present. For this purpose, 15 HIV coinfected and 20 HCV monoinfected patients were compared. They were peg-IFN + RBV non-responders and infected with HCV 1a.

Results: Irrespectively of the HIV-coexistence, at baseline the amino acid variation in the NS5A-related domains was significantly higher than in the E2-PePHD ($p < 0.001$). The number of amino acid variations (mean ± SD) at the NS5A-ISDR domain was higher among HCV/HIV patients than HCV-monoinfected ones (1.80 ± 0.77 vs. 0.95 ± 1.05; $p = 0.009$) but such difference was slightly lower when comparing NS5A-PKRBD sequences (2.47 ± 1.13 vs. 1.60 ± 1.57; $p = 0.06$). No differences were found at the E2-PePHD (0 ± 0 vs. 0.2 ± 0.4). At intra-HIV coinfected patient level, only minor (HCV genetic analysis) or no (HCV substitution rate and quasispecies heterogeneity) changes were observed during therapy (basal, 24 h, 4 weeks, and 12 weeks).

Conclusions: Among HCV-1a/HIV coinfected and HCV-monoinfected peg-IFN + RBV non-responder patients, the HCV variability at the 5′UTR, E2-PePHD and NS5A-PKRBD/ISDR domains was mostly comparable exhibiting a low number of variations. Four well-defined amino acid substitutions in NS5A-ISDR domain appeared most frequently when HIV coexists. The interferon-based therapy did not exert any effect in the variation, selection or diversity in the above mentioned HCV regions that could influence clinical responsiveness to IFN therapy.

1. Introduction

The World Health Organization (WHO) estimates that about 3% of the world's population is infected with hepatitis C virus (HCV) and that there are more than 170 million chronic carriers who are at risk of developing liver cirrhosis and/or liver cancer (Health, 2002). Around 20% of the people living with HIV/aids are coinfected with HCV (7 million persons) (Soriano et al., 2010). In Argentina, the authors have reported that the HIV/HCV coinfection rate rose to 21% (Laufer et al., 2010) being the HCV genotype 1a (HCV-1a) the most frequent (Bolcic et al., 2008b, 2011).

Although protease inhibitors have been approved for HCV genotype 1 treatment in monoinfected patients (Zeuzem et al., 2011), in HIV/HCV coinfected patients pegylated interferon and Ribavirin (peg-IFN + RBV) is still the standard treatment for chronic HCV (Chung et al., 2004). Individuals infected with HCV genotype 1a/b achieved sustained virological response (SVR) in 38–52% of cases. However in HIV coinfected subjects, this treatment results in SVR in only 14–36% (Chung et al., 2004).

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http://dx.doi.org/10.1016/j.antiviral.2012.05.015

Different HCV genomic regions were described as viral factors influencing the response to the combination therapy. Among them, the 5′UTR together with the first 30 nucleotides of the core region acting as an internal ribosome entry site (IRES), the PKR/eIF-2α phosphorylation homology domain (PePHD), included in the E2 glycoprotein and, the interferon sensitivity determining region (ISDR) and the PKR binding domain (PKRBD) into the non-structural 5A (NS5A) protein, were proposed (Le Guillou-Guillemette et al., 2007; Yahoo et al., 2011). The 5′UTR role is yet to be elucidated since different studies showed conflicting results (Dash et al., 2005; Koev et al., 2002). It has been previously described that HCV can replicate in extra-hepatic compartments and that HCV-IRES containing three well-defined nucleotide substitutions have significantly greater translational activity in human lymphoblastoid cell lines (Lerat et al., 2000). The infection and replication in peripheral blood mononuclear cells (PBMC) as a viral sanctuary could be a strategy to evade the HCV treatment allowing the virus to relapse when therapy is suspended (Zignego et al., 1995).

The E2-PePHD has been shown to interact with PKR and inhibit it *in vitro*, suggesting a possible mechanism of HCV to evade the antiviral effects of IFN (Taylor et al., 1999). Though some studies showed that mutations in this region are believed to influence the response to IFN therapy (Gupta et al., 2006; Saito et al., 2003; Sarrazin et al., 2001), other studies could not find any evidence of correlations (Gaudy et al., 2005; Hung et al., 2003; Munoz de Rueda et al., 2008; Vuillermoz et al., 2004).

Another possible mechanism to counteract the antiviral effects of IFN was proposed to be included in the NS5A protein. Enomoto et al. described the ISDR (codons 2209-2248) (Enomoto et al., 1996) and Gale et al. (Gale et al., 1997) showed *in vitro* that the C-terminal codons 2209 and 2274 (PKRBD) of the NS5A protein are able to inhibit the PKR through a direct interaction.

In a previous study our group reported that the number of amino acid mutation in the NS5A-PKRBD and NS5A-ISDR differed between HCV-1a HIV coinfected responder and non responder patients (Laufer et al., 2011).

The aim of this study was to comparatively evaluate the pretreatment HCV-1a variability between non-responder HIV coinfected and HCV monoinfected patients in these four HCV genomic domains as well as to longitudinally assess the impact of peg-IFN + RBV on these HCV genomic regions variability when HIV is co-present.

2. Materials and methods

2.1. Patients and samples

Among 593 HIV-positive patients, >18 years old, who attended for their scheduled controls for HIV viral load (VL) at the Argentinean National Reference Centre for AIDS (CNRS), 15 HIV patients chronically coinfected with HCV genotype 1a followed up prior to and during therapy with peg-IFN + RBV were included. They were non-cirrhotic and non-responders to peg-IFN + RBV therapy. This study was approved by the Ethics Committee and consents were obtained from all participating patients. Patients were treated with peg-IFN + RBV for 12 weeks, because they did not reach early virological response (EVR). Non-adherence to HCV therapy was excluded by the medical practitioners. At baseline, all the subjects exhibit undetectable HIV viral load level (<50 copies/mL) in response to a stable antiretroviral treatment. Blood samples were collected before starting anti-HCV interferon-based therapy and, at 24 h, 4 weeks and 12 weeks during treatment. Plasma samples were aliquoted and conserved at −80 °C until use. PBMCs were obtained by Ficoll-Hypaque density gradient (Gibco BRL, Carlsbad, CA, USA) and carefully washed 3 times in order to ensure removal of surface virus. Plasma HCV-viral load (Bayer VERSANT® HCV RNA 3.0 Assay) and HCV qualitative PCR (Cobas Amplicor HCV 2.0) were evaluated at each time point. The HCV genotype was determined by LiPA VERSANT® HCV Genotype Assay (Siemens Healthcare Diagnostics, Argentina). Basal characteristics of HCV/HIV coinfected patients are represented in Table 1.

2.2. HCV RNA isolation, reverse transcription, amplification, cloning and sequencing

Viral RNA extraction, reverse transcription and amplification were performed as follows: viral RNA extraction was performed from 100 μL plasma or 10^6 PBMCs using a phenol/guanidine isothiocyanate solution (TrizolTM LS Reagent; Gibco BRL, Carlsbad, CA, USA), according to the manufacturer's instructions. The final product was dissolved in 20 μL of RNase-free water. Reverse transcription was performed with MMLV reverse transcriptase and random hexamers. The 5'UTR, E2 and NS5A nested-PCR protocol was performed as previously described (Bolcic et al., 2008a; Moretti et al., 2010) allowing to obtain amplicons with 223 bp (77–300), 319 bp (2214–2532) and 457 bp (6867–7324), respectively. The amplified products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. The DNA of each strain obtained from the purified nested-PCR products (Quick Spin; Qiagen, Venlo, Netherlands) was directly subjected to double-strand sequencing with dye-labeled dideoxy terminators with primers for each region (ABI PRISM 3100 automated sequencer, Applied Biosystems, Foster City, CA, USA). The E2 and NS5A purified PCR-nested products were also cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions, and sequencing was performed.

2.3. HCV sequence analysis

The 5′UTR, E2 and NS5A consensus sequences as determined by directly sequencing were edited and assembled using Sequencher software v.4.10.1 (Gene Codes). Multiple sequence alignment was achieved by the Mafft program (Katoh et al., 2009).

To compare the nucleotide and amino acid mutations, a consensus reference sequence of 222 complete sequences from HCV database (Kuiken et al., 2008) was built. E2 and NS5A nucleotide sequences were toggle translated and the number of amino acid mutations were visually counted.

Basal sequences from 20 HCV-1a monoinfected, non-responders to peg-IFN + RBV therapy from the Virahep-C study (Cannon et al., 2008) were used to compare the pretreatment sequence from HCV monoinfected and HIV coinfected patients (GenBank EU362899 EF407428, EU362900, EF407452, EU362901, EU362902, EU362903, EF407453, EU362904, EF407435, EF407450, EU362905, EU362906, EU362907, EU362908, EF407438, EU362909, EU362910, EU362911, EF407433).

2.4. HCV phylogenetics analysis

Maximum likelihood phylogenetic trees were performed for E2 and NS5A sequences. The most appropriate substitution model to investigate our dataset was the GTR + I + G, selected with the jModelTest 3.7 software. Phylogenetic relationships among HCV-1a strains were estimated with PhyML version 3.0 computer program. Phylogenetic consistency of individual nodes in the most likely tree topology was estimated with a non-parametric bootstrap test (100 replicates). The tree topologies were midpoint rooted for illustration purposes and drawn using the Dendroscope v2.7.4 program.

Table 1
Basal characteristics of the 11 HCV-1a/HIV coinfected non responder patients The number of nucleotide (for 5′UTR) and amino acid (for PePHD, PePHD flanking region, ISDR, and PKRBD) variations are also shown.

Patient	Age (Years)	Weight (Kilograms)	CD4 (Cells/ml)[a]	ALT (U/L)[a]	Gender	Risk Group	Sample	HCV-VL (log IU/ml)	5′UTR	E2		NS5A	
									IRES	PePHD	PePHD Flanking Regions	ISDR	PKRBD[b]
1	47	73	400	49	M	IDU	Basal	5.7	0	0	1	2	0
							24h	6.0	0	0	1	2	0
							week4	5.7	0	0	1	2	0
							week12	5.8	0	0	1	2	0
2	37	77	522	66	M	IDU	Basal	6.0	3	0	5	3	1
							24h	5.1	4	0	4	3	1
							week4	5.2	3	0	4	3	1
							week12	5.0	3	0	4	3	1
3	35	67	817	99	M	IDU	Basal	6.4	0	0	3	2	1
							24h	5.7	0	0	3	2	1
							week4	5.0	0	0	3	1	1
							week12	6.0	0	0	3	2	1
4	39	76	414	48	M	IDU	Basal	5.6	2	0	6	2	1
							24h	5.2	2	0	6	2	1
							week4	4.6	2	0	7	2	1
							week12	3.5	2	0	6	2	1
5	42	83	130	60	M	IDU	Basal	6.4	0	0	2	2	0
							24hs	5.9	0	0	2	1	0
							week4	4.6	0	0	3	2	1
							week12	5.3	0	0	4	2	1
7	33	64	479	53	M	IDU	Basal	4.69	0	0	4	1	2
							24hs	4.71	0	0	4	1	2
							week4	4.7	0	0	4	1	2
							week12	4.5	0	0	4	1	2
9	51	65	625	105	M	HTS	Basal	6.7	0	0	4	0	0
							24h	6.1	0	0	4	0	0
							week4	5.9	0	0	4	0	0
							week12	5.1	0	0	4	1	0
10	40	75	303	97	M	IDU	Basal	5.9	0	0	1	1	0
							24h	5.8	0	0	1	1	0
							week4	5.5	0	0	1	1	0
							week12	5.2	0	0	1	1	0
11	38	50	644	59	F	IDU	Basal	6.0	1	0	1	2	0
							24hs	5.8	1	0	1	2	0
							week4	4.7	1	0	1	2	1
							week12	5.8	1	0	1	3	0
15	45	70	383	50	M	MSM	Basal	5.397	1	0	2	2	0
							24hs	5.028	1	0	2	2	0
							week4	4.215	1	0	2	2	0
							week12	4.299	1	0	2	2	0
16	41	58	262	92	M	MSM	Basal	6.5	1	0	2	2	0
							24h	5.4	1	0	2	2	0
							week4	5.1	1	0	2	2	0
							week12	5.2	1	0	2	2	0
17	42	81	957	191	M	IDU	Basal	5.4	0	0	7	3	1
							24h	3.9	0	0	7	3	1
							week4	3.1	0	0	7	3	1
							week12	5.3	0	0	7	4	1
18	45	65	989	115	M	HTS	Basal	4.761	1	0	2	3	1
							24h	3.796	3	0	2	3	1
							week4	4.015	1	0	2	3	1
							week12	3.46	1	0	2	3	1
19	42	95	ND	59	M	IDU	Basal	6.4	1	0	1	2	0
							24h	6.3	1	0	1	2	0
							week4	5.9	1	0	1	2	0
							week12	5.7	1	0	1	2	0
20	40	72	842	215	M	IDU	Basal	6.008	0	0	2	2	1
							24h	5.348	0	0	2	2	1
							week4	4.762	0	0	1	2	1
							week12	4.342	0	0	2	2	1
Mean (SD)	41.3 (4.5)	71.4 (10.8)	554.8 (266.6)	90.5 (51.1)				5.8 (0.6)[a]	0.72 (1)	0	2.8 (1.9)	1.9 (0.8)	0.6 (0.6)

IDU: injecting drug user; HTS: heterosexual; MSM: men who have sex with men.
M: male; F: female.
ND: not determined.
[a]Number of mutations in the 26 amino acids adjacent to the ISDR.
[b]At baseline.

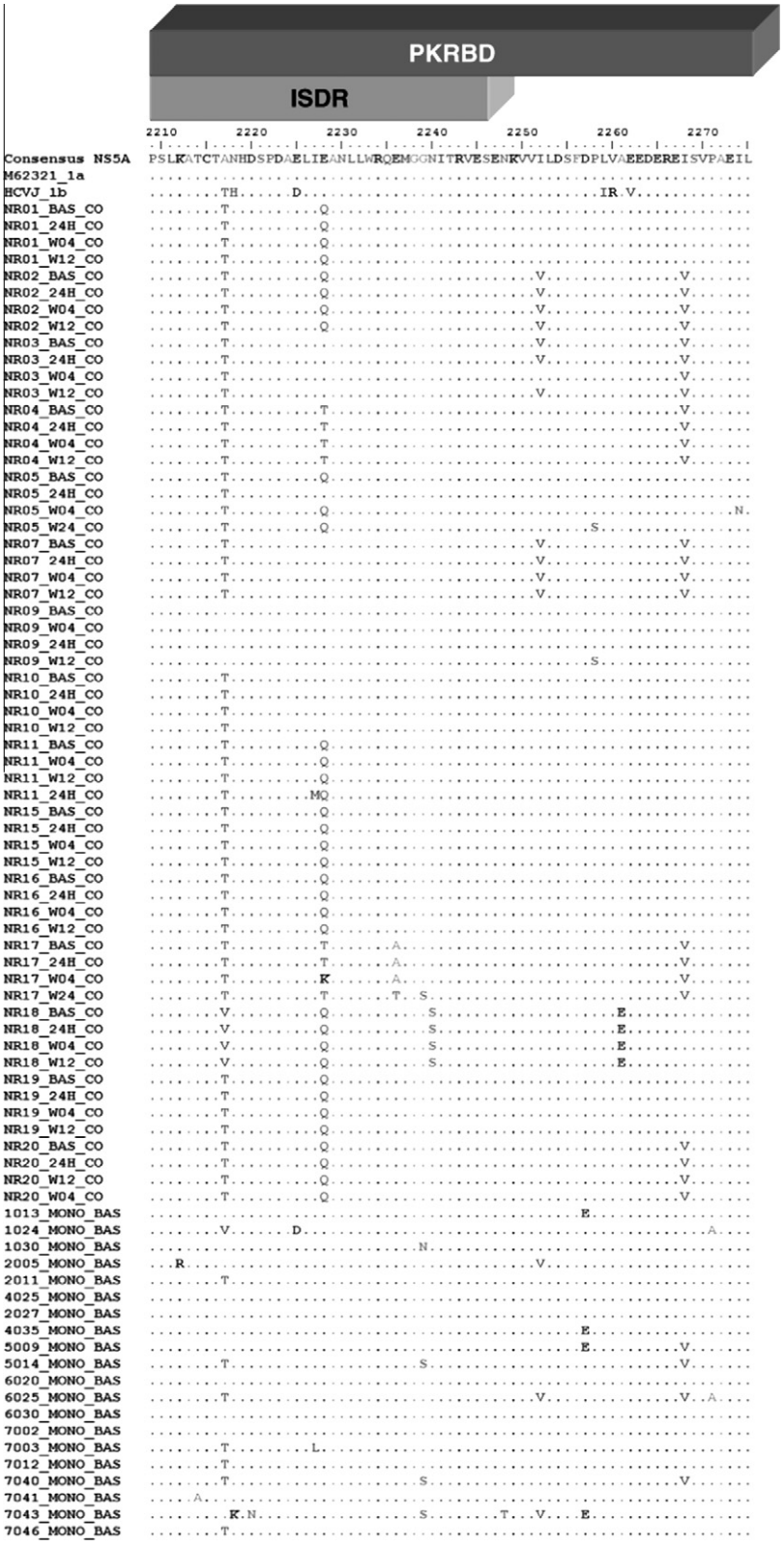

Fig. 1. HCV-ISDR and PKRBD amino acids sequences aligned to the consensus sequence, from non responders HCV/HIV coinfected patients before (basal) and during peg-IFN + RBV treatment (24 h, 4 weeks and 12 weeks) and sequences before treatment obtained from HCV monoinfected patients from the Virahep study (Cannon et al., 2008). Reference sequences M62321 and D90208 (HCV-J) are also shown.

2.5. HCV nucleotide substitution rates

The estimation of nucleotide substitutions rates per site per year based on E2 and NS5A nucleotide sequences from 15 HIV/HCV coinfected patients was performed using the Bayesian Markov chain Monte Carlo (MCMC) algorithm implemented in the BEAST v1.5.4 program at different sampling times (baseline, 24 h, 4 weeks and 12 weeks during therapy). Analyses were run twice for 10^6 generations, sampling tree every 1000 steps and discarding the first 10% as burn-in. MCMC convergence and effective sample size (ESS) were checked using the TRACER v1.5 program. Analyses were considered to have converged and reached stability after the burn-in period when ESS were higher than 200. Uncertainty in parameter estimates was evaluated in 95% of the highest posterior density (HPD 95%) interval.

2.6. Inter-patient HCV diversity and positive selection

In order to compare the HCV genetic diversity from HIV-coinfected patients before and during treatment, the HCV genetic distance was estimated using the MEGA 4.0 software. The HCV quasispecies complexity was measured by calculating the normalized Shannon entropy (Sn) based on both E2 and NS5A clone-derived sequence data.

To determine the degree of the positive selection in HCV nucleotide sequences from HIV coinfected patients, the alignments of the NS5A and the E2 genomic region at different sampling times during the follow-up were analyzed using the single likelihood ancestor counting (SLAC) algorithm. The positive selection analysis was carried out using the HyPhy program.

2.7. Statistical analysis

The statistical analysis was performed using the Chi-square and Fisher's exact test. The analysis of continuous variables was performed using the Student *t*-test. A p value <0.05 was considered significant. All analyses were performed using the SPSS version 12.0 (SPSS, Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

After matching both patient-related (age, gender, weight, fibrosis stage) and HCV-related (plasma viral load) characteristics be-

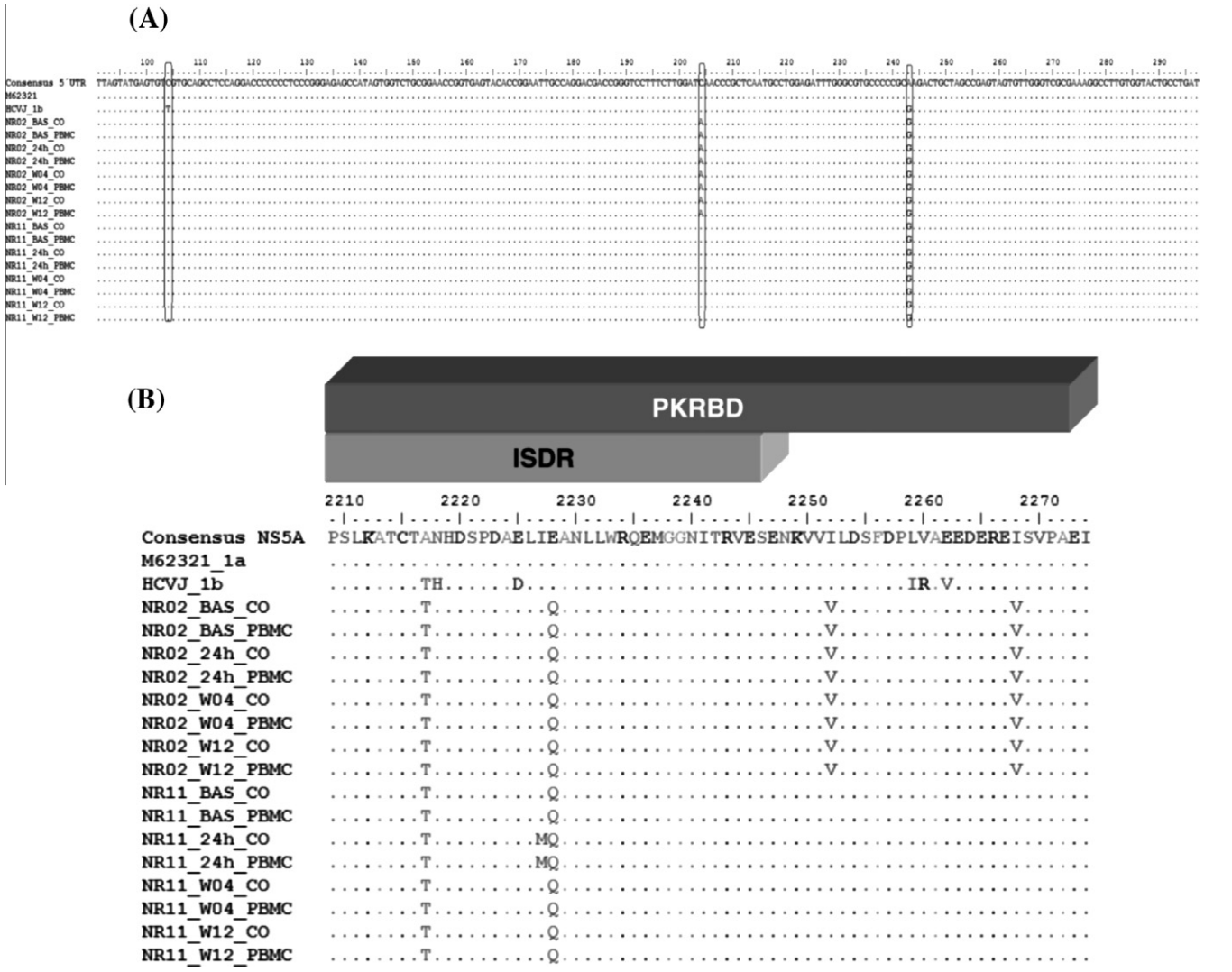

Fig. 2. HCV 5'UTR (A) and NS5A ISDR/PKRBD (B) sequences comparison between both, plasma (CO) and PBMC compartment during the follows-up (Basal, 24 h, 4 weeks and 12 weeks). In (A), the boxes represent the three positions associated to lymphotropism.

tween the HIV/HCV coinfected group and the HCV-1a monoinfected group recruited in the Virahep-C study (Cannon et al, 2008), no significant differences ($p > 0.05$) were found. No data on CD4 T cell counts were available in the latter group.

3.2. Inter-patient comparative HCV sequence analysis at baseline

At baseline, HIV coinfected and HCV monoinfected patients presented high amino acid conservation in the E2-PePHD. The mean number of amino acid changes (±SD) were 0 (±0) and 0.2 (±0.4), respectively. When comparing them, no significant differences were founded ($p = 0.15$). However, the E2-PePHD flanking regions exhibited a pattern of variations characteristic for each one (Supplementary Fig. S1). On the other hand, the number of amino acid mutations in the NS5A-ISDR was statistically higher in HIV coinfected patients than in monoinfected ones (1.80 ± 0.77 vs. 0.95 ± 1.05; $p = 0.009$), but it was just slightly higher at the NS5A-PKBD (2.47 ± 1.13 vs. 1.60 ± 1.57; $p = 0.08$). The A2217T, I2252V, and I2268V mutations at the NS5A-ISDR/PKRBD were the most frequently detected among HIV coinfected and HCV monoinfected patients, while another (E2228Q) was only present among HCV isolates from HIV coinfected patients (Fig. 1). These four mutations were significantly more frequent among HCV isolates from HIV coinfected patients (28 out of 31 amino acidic changes) than among HCV-monoinfected individuals (15 out of 32 amino acid mutations) [$p = 0.0047$].

Independently of HIV coinfection, the number of amino acid mutation in the NS5A-ISDR and NS5A-PKRBD was significantly higher than in E2-PePHD ($p < 0.001$).

No HCV isolates exhibited simultaneously the three IRES-5′UTR mutations (G107A, C204A, G243A) associated with greater capacity for replication due to more efficient cap-independent translation in lymphoid cells (Laskus et al., 2000; Lerat et al., 2000). Only the mutation G243A was observed in 2 out of 15 HIV coinfected patients (Supplementary Fig. S3) but they did not exhibit a higher level of plasma viral load (Table 1). Furthermore, the intra-patient analysis at 5′UTR and NS5A sequences during the follow-up from plasma and PBMCs in these two patients, exhibited no differences at the nucleotide or amino acid level of these regions, respectively (Fig. 2).

The HCV phylogenetic analysis by maximum likelihood based on E2 and NS5A nucleotide sequences from both groups of non-responder patients appears intermingled evidencing no clustering phenomenon when HIV coexists, and indicating that they are not derived from separate evolutionary lineages (Fig. 3).

3.3. Intra-patient analysis of HCV sequences during peg-IFN+RBV therapy

The previous measures of inter-patient HCV diversity can be influenced by variations in the external reference sequence, although our strategy to use a consensus sequence obtained from 222 HCV-1a sequences from database was to dampen this concern related to using an arbitrary isolate as a reference. Therefore, the genetic variation among the HCV sequences was assessed by two additional measures that are not dependent upon an external reference sequence, genetic distance and Shannon's entropy based on E2 and NS5A clone-derived sequences from HIV coinfected patients.

HCV-E2 and NS5A sequences from each HIV coinfected patient were evaluated during peg-IFN + RBV therapy. The E2-PePHD amino acid sequences remained unchanged under treatment whereas the PePHD flanking regions exhibited mutations characteristics for each patient that remained unchanged during HCV therapy (Supplementary Fig. S1). Nevertheless, the HCV plasma viral load exhibited not differences when comparing it among patients at each sampling time (Table 1).

The number of ISDR and PKRBD amino acid substitutions did not present significant differences during the follow-up ($p > 0.05$), suggesting that the peg-IFN + RBV did no promote the emergence of new mutations such as are seen with antiviral therapies which target the active site of a viral enzyme (Fig. 1).

The phylogenetic inferences showed that HCV E2 and NS5A sequences obtained from a given patient were more closely related among themselves in comparison with those from other patients, generating a patient's cluster with a monophyletic origin (Fig. 3).

The HCV nucleotide substitution rates (95% HPD) based on the E2 and NS5A sequence analysis were 2.3×10^{-3} (5×10^{-9}–1.3×10^{-3}) and 7×10^{-3} (2.1×10^{-7}–1.8×10^{-3}), respectively. These rates remained almost unchanged when the intra-patient estimation was performed at each sampling time. The substitution rates (95% HPD) at 24 h, 4 weeks and 12 weeks were 2.3×10^{-3} (3.6×10^{-8}–5.8×10^{-4}); 2.3×10^{-3} (2.3×10^{-8}–5.4×10^{-4}) and 4×10^{-3} (6.2×10^{-8}–1.1×10^{-3}) for E2 region and, 8.5×10^{-3} (2.7×10^{-7}–1.4×10^{-3}); 8.5×10^{-3} (6.8×10^{-7}–1.9×10^{-3}) and 6.6×10^{-3} (4.48×10^{-7}–1.7×10^{-3}) for NS5A, respectively.

The HCV quasispecies analysis exhibited higher heterogeneity (measured as Shannon entropy) when based on NS5A-PKRBD sequences compared with E2-PePHD sequences (0.88 ± 0.12 and 0.23 ± 0.17, respectively [$p < 0.05$]). During peg-IFN + RBV therapy, such heterogeneity was not affected ($p > 0.05$). Of note, the E2-PePHD region is smaller than the NS5A-PKRBD, so the results exposed here could be related with the differences in the number of nucleotide analyzed.

No positive selection occurred during treatment in both E2-PePHD and NS5A-PKRBD regions.

4. Discussion

The present study was aimed to determine whether the numbers of mutations in the 5′UTR-IRES, E2-PePHD, NS5A-ISDR, and NS5A-PKRBD regions isolated from the pretreatment serum samples of peg-IFN + RBV non-responder HCV-1a chronically infected patients were different when HIV coexists. A longitudinal intra-patient analysis of the HCV genomic diversity based on these four domains was also assessed among those HIV coinfected ones. The majority of the previous studies were done with a lower number of HCV-1a infected non-responder patients, and using different treatments of peg-IFN + RBV. Moreover, the polymorphisms in the viral RNA 5′UTR -IRES have been less widely investigated. Nevertheless, in previous studies, no significant difference between the polymorphisms in this region and the response to IFN was found (Soler et al., 2002; Thelu et al., 2004).

The 5′UTR-IRES, E2-PePHD, and NS5A-ISDR/PKRBD pretreatment sequences from HCV-1a isolates did not exhibit substantial genomic variability between non-responder HIV coinfected and HCV monoinfected patients, without evidence of phylogenetic cluster association. These results denote that the HCV-1a strains from monoinfected and HIV coinfected patients did not diverge in their origin as well as that the viral factors studied did not offer an argument to explain the lower rate of therapy response extensively reported (Arends et al., 2011; Carrat et al., 2004; Chung et al., 2004; Nunez et al., 2007; Torriani et al., 2004) among HIV coinfected patients.

It is still controversial the role of the extrahepatic replication of HCV in mononuclear cells as well as its role as a contributive strategy to IFN-based HCV treatment unresponsiveness (Gil et al., 1993; Saleh et al., 1994). The HCV strains characterized in mononuclear cells from HIV coinfected patients did not appear to exhibit greater

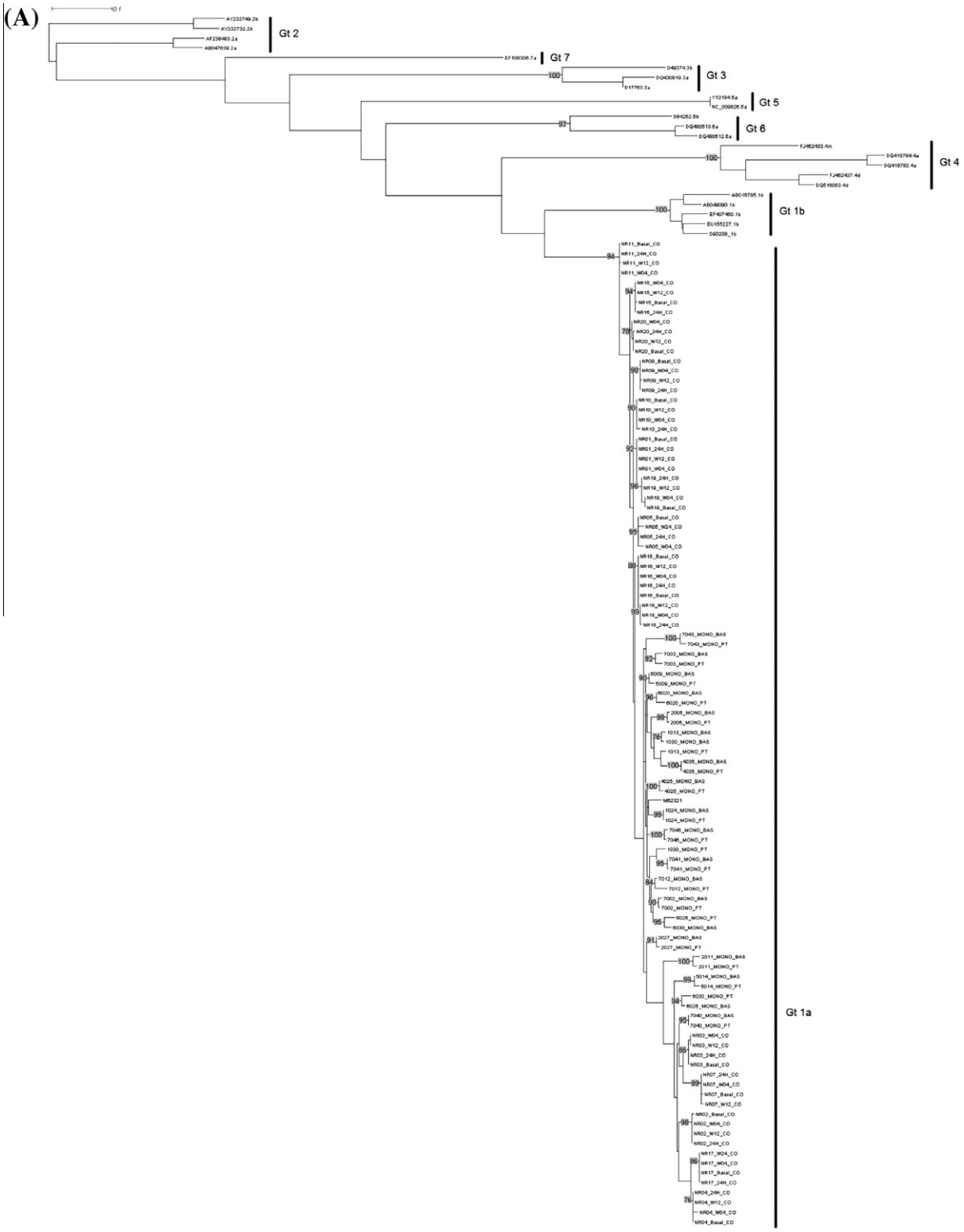

Fig. 3. Maximum likelihood tree of the HCV E2 (A) and NS5A (B) region. Isolates from HIV coinfected patients before (basal) and during treatment (24 h, 4 weeks and 12 weeks) and from HCV monoinfected ones before treatment are represented. The HIV coinfected sequences are denoted with the non-responder (NR) patient number followed by the sampling time; the HCV sequences from Virahep-C study are identified with the name of the HCV isolate as denoted in Cannon et al. (2008). Bootstrap values are shown on branches. Branch lengths are proportional to the number of nucleotide substitutions per aligned site (bar = 0.1 substitutions).

capacity for viral replication in lymphoid cells because the associated mutations on the 5′UTR-IRES were infrequently found. In this regard, there are two limitations: few HCV isolates were characterized on PBMC and, only the positive-strand HCV RNA was investigated. In this context, the level of replication within the PBMCs among HIV coinfected individuals would be low and most of HCV might be present on the cell surface, as recently reported (Natarajan et al., 2010) but a final conclusion needs further investigation. Likewise, the HCV genomic characterization based on NS5A revealed no differences neither between paired samples from both

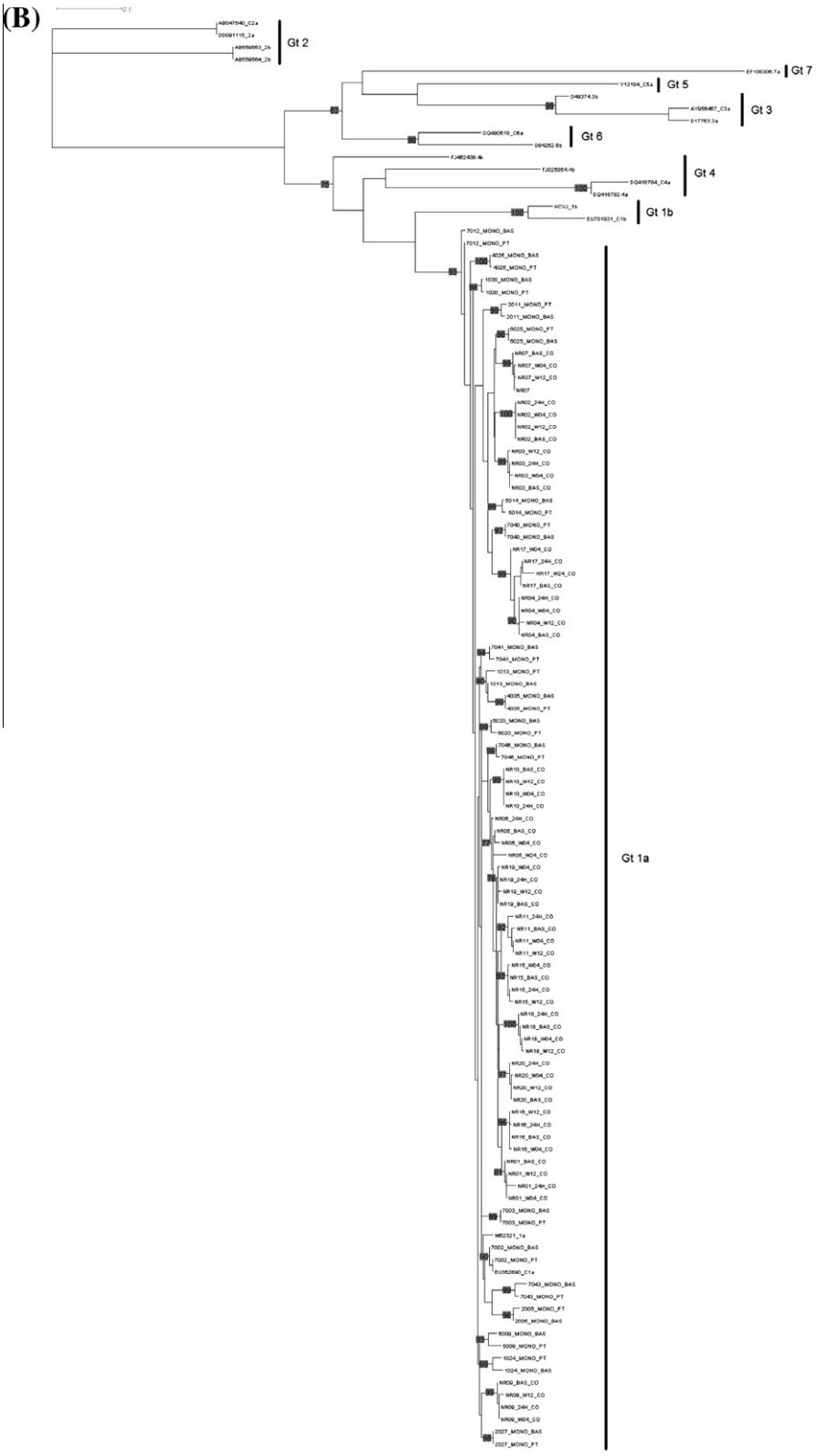

Fig. 3 (*continued*)

compartments nor among successive samples in a given compartment. But the number of HCV isolates characterized in PBMCs from non-responder patients is too small to draw definitive conclusions.

The present study shows that HCV-1a E2-PePHD was highly conserved at both inter-patient level and intra-patient during peg-IFN + RBV therapy. In agreement with several previous reports (Chayama et al., 2000; Hung et al., 2003; Sarrazin et al., 2001) but in contrast with others (Afzal et al., 2010; Ukai et al., 2006) there are no significant correlations between the substitutions in E2-PePHD and the absence of response to IFN-based therapy. However, these studies were carried out among HCV-1 monoinfected patients; only one study was reported in patients coinfected with

HIV and HCV-genotype 3a showing that the region E2-PePHD was highly conserved, with no correlation to the anti-HCV treatment response (Bagaglio et al., 2003). Despite the flanking region of E2-PePHD was proposed to correlate with both the response to IFN monotherapy and viral load (Ukai et al., 2006), we did not find these correlations in the flanking region irrespectively of the HIV coexistence.

It has been suggested that the NS5A protein plays a critical role in HCV-RNA replication where certain mutations in the ISDR have special incumbency (Kohashi et al., 2006). For the antiviral response to IFN it is likely that NS5A specific amino acid variations – not just the total number of mutations – could be sufficient to impede the PKRBD's interaction with PKR as well as to modulate HCV-RNA replication. Regardless of HIV coinfection, we have consistently found the A2217T, I2252V, and I2268V mutations at the NS5A-ISDR/PKRBD but the E2228Q mutations was only identified among HIV-coinfected subjects. The first two were previously explored *in vitro* (Gale et al., 1998; Macdonald and Harris, 2004; Podevin et al., 2001) and *in vivo* (Murphy et al., 2002; Yahoo et al., 2011) in their impact on NS5A-PKR interaction. Nevertheless, the precise mechanistic effect of the E2228Q and I2268V amino acid substitutions on protein function and IFN resistance remains to be elucidated.

The NS5A-ISDR and PKRBD are useful as predictive markers of the response to IFN therapy for patients with a Japanese-specific subtype (HCV-J) (Enomoto et al., 1996; Murayama et al., 2007), but similar correlations were not observed in studies conducted in Europe and the United States (Gerotto et al., 1999; Murphy et al., 2002; Zeuzem et al., 1997). Nevertheless, a meta-analysis of 655 Japanese and 525 European patients focused on geographical differences (Pascu et al., 2004) concluded that there was a significant positive correlation between the number of NS5A domains mutations and the sustained response rate, irrespective of the geographical region. Here we found that the mean number of amino acid mutations in NS5A-ISDR and PKRBD in non-responder patients was less than two and three, respectively regardless of HIV infection, and the number of mutations remained unchanged during 12 weeks of treatment in HIV coinfected patients.

In the evolutive study, we observed for the non-responder HIV-coinfected patients that the E2-PePHD, NS5A-ISDR and NS5A-PKRBD regions did not present significant changes so that the predominant quasispecies of the pretreatment were persistent during treatment.

It is worth noting that these studies were performed only with partial sequences of three genomic regions, thereby to obtain a definitive conclusion imposes studies with complete sequences of the virus. In turn, the number of HCV isolates from each group should be increased so as to increase the probability of recognition of related sequences.

When comparing HCV-monoinfected and HCV/HIV-coinfected patients no differences were found in several host-related factors that could impact on therapy outcome (age, gender, weight, fibrosis stage, alanine-amino transferase –ALT-). A clear correlation between increased HCV genetic diversity and CD4 cell counts in HCV/HIV co-infected patients with significant immune reconstitution was reported (Wang et al., 2006). The CD4 + T cell count was only available for the latter at baseline and could not be adequately addressed in this study. Also, many single nucleotide polymorphisms (SNPs) associated with outcome of interferon-based therapy have been identified (Abe et al., 2011) and need further evaluation.

5. Conclusions

Among HCV-1a/HIV coinfected and HCV-monoinfected peg-IFN + RBV non-responder patients, the HCV variability at the 5'UTR, E2-PePHD and NS5A-PKRBD/ISDR domains were mostly comparable. The overall number of variations in these regions was similarly limited but well-defined amino acid substitutions in NS5A-ISDR domain appeared most frequently when HIV coexists. The interferon-based therapy did not exert any effect in the variation, selection or diversity in the above mentioned HCV regions that could influence clinical responsiveness to IFN therapy.

Disclosure

The authors have no conflict(s) of interest

Acknowledgments

This study was supported partially by grants from the University of Buenos Aires (SECYT-UBA 2010–2012), and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET-PIP112 200801 01773). The authors thank Sergio Mazzini (Centro Nacional de Referencia para el SIDA, Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Argentina) for his assistance during the manuscript preparation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.05.015.

References

Abe, H., Hayes, C.N., Ochi, H., Maekawa, T., Tsuge, M., Miki, D., Mitsui, F., Hiraga, N., Imamura, M., Takahashi, S., Kubo, M., Nakamura, Y., Chayama, K., 2011. IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy. J. Hepatol. 54, 1094–1101.

Afzal, S., Idrees, M., Akram, M., Awan, Z., Khubaib, B., Aftab, M., Fatima, Z., Badar, S., Hussain, A., 2010. Mutations in the E2-PePHD region of hepatitis C virus genotype-3a and correlation with response to interferon and ribavirin combination therapy in Pakistani patients. Virol. J. 7, 377.

Arends, J.E., van Assen, S., Stek, C.J., Wensing, A.M., Fransen, J.H., Schellens, I.M., Spijkers, S.N., Mudrikova, T., van Baarle, D., Sprenger, H.G., Hoepelman, A.I., 2011. Pegylated interferon-alpha monotherapy leads to low response rates in HIV-infected patients with acute hepatitis C. Antivir. Ther. 16, 979–988.

Bagaglio, S., Bruno, R., Lodrini, S., De Mitri, M.S., Andreone, P., Loggi, E., Galli, L., Lazzarin, A., Morsica, G., 2003. Genetic heterogeneity of hepatitis C virus (HCV) in clinical strains of HIV positive and HIV negative patients chronically infected with HCV genotype 3a. J. Biol. Regul. Homeost. Agents 17, 153–161.

Bolcic, F., Bull, L., Martinez, L., Reynoso, R., Salomon, H., Arduino, R., Barnett, B., Quarleri, J., 2008a. Analysis of sequence configurations of the PKR-interacting HCV proteins from plasma and PBMC as predictors of response to interferon-alpha and ribavirin therapy in HIV-coinfected patients. Intervirology 51, 261–264.

Bolcic, F., Jones, L., Laufer, N., Quarleri, J., 2011. Molecular characterization of hepatitis C virus genotype 4 sequences in HIV-coinfected patients from Argentina. J. Med. Virol..

Bolcic, F., Laufer, N., Quarleri, J., 2008b. Hepatitis C virus genotypes distribution and viral load level among HIV-coinfected patients. Acta Gastroenterol. Latinoam. 38, 307.

Cannon, N.A., Donlin, M.J., Fan, X., Aurora, R., Tavis, J.E., 2008. Hepatitis C virus diversity and evolution in the full open-reading frame during antiviral therapy. PLoS One 3, e2123.

Carrat, F., Bani-Sadr, F., Pol, S., Rosenthal, E., Lunel-Fabiani, F., Benzekri, A., Morand, P., Goujard, C., Pialoux, G., Piroth, L., Salmon-Ceron, D., Degott, C., Cacoub, P., Perronne, C., 2004. Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial. JAMA 292, 2839–2848.

Chayama, K., Suzuki, F., Tsubota, A., Kobayashi, M., Arase, Y., Saitoh, S., Suzuki, Y., Murashima, N., Ikeda, K., Takahashi, N., Kinoshita, M., Kumada, H., 2000. Association of amino acid sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. Hepatology 32, 1138–1144.

Chung, R.T., Andersen, J., Volberding, P., Robbins, G.K., Liu, T., Sherman, K.E., Peters, M.G., Koziel, M.J., Bhan, A.K., Alston, B., Colquhoun, D., Nevin, T., Harb, G., van der Horst, C., 2004. Peginterferon Alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. N. Engl. J. Med. 351, 451–459.

Dash, S., Prabhu, R., Hazari, S., Bastian, F., Garry, R., Zou, W., Haque, S., Joshi, V., Regenstein, F.G., Thung, S.N., 2005. Interferons alpha, beta, gamma each inhibit hepatitis C virus replication at the level of internal ribosome entry site-mediated translation. Liver Int. 25, 580–594.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F., Sato, C., 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334, 77–81.

Gale Jr., M., Blakely, C.M., Kwieciszewski, B., Tan, S.L., Dossett, M., Tang, N.M., Korth, M.J., Polyak, S.J., Gretch, D.R., Katze, M.G., 1998. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol. Cell Biol. 18, 5208–5218.

Gale Jr., M.J., Korth, M.J., Tang, N.M., Tan, S.L., Hopkins, D.A., Dever, T.E., Polyak, S.J., Gretch, D.R., Katze, M.G., 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230, 217–227.

Gaudy, C., Lambele, M., Moreau, A., Veillon, P., Lunel, F., Goudeau, A., 2005. Mutations within the hepatitis C virus genotype 1b E2-PePHD domain do not correlate with treatment outcome. J. Clin. Microbiol. 43, 750–754.

Gerotto, M., Dal Pero, F., Sullivan, D.G., Chemello, L., Cavalletto, L., Polyak, S.J., Pontisso, P., Gretch, D.R., Alberti, A., 1999. Evidence for sequence selection within the non-structural 5A gene of hepatitis C virus type 1b during unsuccessful treatment with interferon-alpha. J. Viral Hepat. 6, 367–372.

Gil, B., Qian, C., Riezu-Boj, J.I., Civeira, M.P., Prieto, J., 1993. Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C: different patterns of response to interferon treatment. Hepatology 18, 1050–1054.

Gupta, R., Subramani, M., Khaja, M.N., Madhavi, C., Roy, S., Habibullah, C.M., Das, S., 2006. Analysis of mutations within the 5′ untranslated region, interferon sensitivity region, and PePHD region as a function of response to interferon therapy in hepatitis C virus-infected patients in India. J. Clin. Microbiol. 44, 709–715.

Health, N.I.o., 2002. National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002–June 10–12. Hepatology 36, S3–S20.

Hung, C.H., Lee, C.M., Lu, S.N., Lee, J.F., Wang, J.H., Tung, H.D., Chen, T.M., Hu, T.H., Chen, W.J., Changchien, C.S., 2003. Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. J. Viral Hepat. 10, 87–94.

Katoh, K., Asimenos, G., Toh, H., 2009. Multiple alignment of DNA sequences with MAFFT. Methods Mol. Biol. 537, 39–64.

Koev, G., Duncan, R.F., Lai, M.M., 2002. Hepatitis C virus IRES-dependent translation is insensitive to an eIF2alpha-independent mechanism of inhibition by interferon in hepatocyte cell lines. Virology 297, 195–202.

Kohashi, T., Maekawa, S., Sakamoto, N., Kurosaki, M., Watanabe, H., Tanabe, Y., Chen, C.H., Kanazawa, N., Nakagawa, M., Kakinuma, S., Yamashiro, T., Itsui, Y., Koyama, T., Enomoto, N., Watanabe, M., 2006. Site-specific mutation of the interferon sensitivity-determining region (ISDR) modulates hepatitis C virus replication. J. Viral Hepat. 13, 582–590.

Kuiken, C., Hraber, P., Thurmond, J., Yusim, K., 2008. The hepatitis C sequence database in Los Alamos. Nucleic Acids Res. 36, D512–D516.

Laskus, T., Radkowski, M., Piasek, A., Nowicki, M., Horban, A., Cianciara, J., Rakela, J., 2000. Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: evidence of active replication in monocytes/macrophages and lymphocytes. J. Infect. Dis. 181, 442–448.

Laufer, N., Bolcic, F., Rolon, M.J., Martinez, A., Reynoso, R., Perez, H., Salomon, H., Cahn, P., Quarleri, J., 2011. HCV RNA decline in the first 24h exhibits high negative predictive value of sustained virologic response in HIV/HCV genotype 1 co-infected patients treated with peginterferon and ribavirin. Antiviral Res. 90, 92–97.

Laufer, N., Quarleri, J., Bouzas, M.B., Juncos, G., Cabrini, M., Moretti, F., Bolcic, F., Fernandez-Giuliano, S., Mammana, L., Salomon, H., Cahn, P., 2010. Hepatitis B virus, hepatitis C virus and HIV coinfection among people living with HIV/AIDS in Buenos Aires, Argentina. Sex. Transm. Dis. 37, 342–343.

Le Guillou-Guillemette, H., Vallet, S., Gaudy-Graffin, C., Payan, C., Pivert, A., Goudeau, A., Lunel-Fabiani, F., 2007. Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. World J. Gastroenterol. 13, 2416–2426.

Lerat, H., Shimizu, Y.K., Lemon, S.M., 2000. Cell type-specific enhancement of hepatitis C virus internal ribosome entry site-directed translation due to 5′ nontranslated region substitutions selected during passage of virus in lymphoblastoid cells. J. Virol. 74, 7024–7031.

Macdonald, A., Harris, M., 2004. Hepatitis C virus NS5A: tales of a promiscuous protein. J. Gen. Virol. 85, 2485–2502.

Moretti, F., Bolcic, F., Mammana, L., Bouzas, M.B., Laufer, N., Quarleri, J., 2010. The hepatitis C virus 5′UTR genomic region remains highly conserved under HAART: a 4- to 8-year longitudinal study from HCV/HIV co-infected patients. AIDS Res. Hum. Retroviruses 26, 527–532.

Munoz de Rueda, P., Casado, J., Paton, R., Quintero, D., Palacios, A., Gila, A., Quiles, R., Leon, J., Ruiz-Extremera, A., Salmeron, J., 2008. Mutations in E2-PePHD, NS5A-PKRBD, NS5A-ISDR, and NS5A-V3 of hepatitis C virus genotype 1 and their relationships to pegylated interferon-ribavirin treatment responses. J. Virol. 82, 6644–6653.

Murayama, M., Katano, Y., Nakano, I., Ishigami, M., Hayashi, K., Honda, T., Hirooka, Y., Itoh, A., Goto, H., 2007. A mutation in the interferon sensitivity-determining region is associated with responsiveness to interferon-ribavirin combination therapy in chronic hepatitis patients infected with a Japan-specific subtype of hepatitis C virus genotype 1B. J. Med. Virol. 79, 35–40.

Murphy, M.D., Rosen, H.R., Marousek, G.I., Chou, S., 2002. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. Dig. Dis. Sci. 47, 1195–1205.

Natarajan, V., Kottilil, S., Hazen, A., Adelsberger, J., Murphy, A.A., Polis, M.A., Kovacs, J.A., 2010. HCV in peripheral blood mononuclear cells are predominantly carried on the surface of cells in HIV/HCV co-infected individuals. J. Med. Virol. 82, 2032–2037.

Nunez, M., Marino, A., Miralles, C., Berdun, M.A., Sola, J., Hernandez-Burruezo, J.J., Galindo, M.J., Barreiro, P., Martin-Carbonero, L., Soriano, V., 2007. Baseline serum hepatitis C virus (HCV) RNA level and response at week 4 are the best predictors of relapse after treatment with pegylated interferon plus ribavirin in HIV/HCV-coinfected patients. J. Acquir. Immune. Defic. Syndr. 45, 439–444.

Pascu, M., Martus, P., Hohne, M., Wiedenmann, B., Hopf, U., Schreier, E., Berg, T., 2004. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut 53, 1345–1351.

Podevin, P., Sabile, A., Gajardo, R., Delhem, N., Abadie, A., Lozach, P.Y., Beretta, L., Brechot, C., 2001. Expression of hepatitis C virus NS5A natural mutants in a hepatocytic cell line inhibits the antiviral effect of interferon in a PKR-independent manner. Hepatology 33, 1503–1511.

Saito, T., Ito, T., Ishiko, H., Yonaha, M., Morikawa, K., Miyokawa, A., Mitamura, K., 2003. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon therapy in Japanese patients infected with HCV genotypes 2a and 2b. Am. J. Gastroenterol. 98, 1377–1383.

Saleh, M.G., Tibbs, C.J., Koskinas, J., Pereira, L.M., Bomford, A.B., Portmann, B.C., McFarlane, I.G., Williams, R., 1994. Hepatic and extrahepatic hepatitis C virus replication in relation to response to interferon therapy. Hepatology 20, 1399–1404.

Sarrazin, C., Bruckner, M., Herrmann, E., Ruster, B., Bruch, K., Roth, W.K., Zeuzem, S., 2001. Quasispecies heterogeneity of the carboxy-terminal part of the E2 gene including the PePHD and sensitivity of hepatitis C virus 1b isolates to antiviral therapy. Virology 289, 150–163.

Soler, M., Pellerin, M., Malnou, C.E., Dhumeaux, D., Kean, K.M., Pawlotsky, J.M., 2002. Quasispecies heterogeneity and constraints on the evolution of the 5′ noncoding region of hepatitis C virus (HCV): relationship with HCV resistance to interferon-alpha therapy. Virology 298, 160–173.

Soriano, V., Vispo, E., Labarga, P., Medrano, J., Barreiro, P., 2010. Viral hepatitis and HIV co-infection. Antiviral Res. 85, 303–315.

Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N., Lai, M.M., 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.

Thelu, M.A., Drouet, E., Hilleret, M.N., Zarski, J.P., 2004. Lack of clinical significance of variability in the internal ribosome entry site of hepatitis C virus. J. Med. Virol. 72, 396–405.

Torriani, F.J., Rodriguez-Torres, M., Rockstroh, J.K., Lissen, E., Gonzalez-Garcia, J., Lazzarin, A., Carosi, G., Sasadeusz, J., Katlama, C., Montaner, J., Sette Jr., H., Passe, S., De Pamphilis, J., Duff, F., Schrenk, U.M., Dieterich, D.T., 2004. Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. N. Engl. J. Med. 351, 438–450.

Ukai, K., Ishigami, M., Yoshioka, K., Kawabe, N., Katano, Y., Hayashi, K., Honda, T., Yano, M., Goto, H., 2006. Mutations in carboxy-terminal part of E2 including PKR/eIF2alpha phosphorylation homology domain and interferon sensitivity determining region of nonstructural 5A of hepatitis C virus 1b: their correlation with response to interferon monotherapy and viral load. World J. Gastroenterol. 12, 3722–3728.

Vuillermoz, I., Khattab, E., Sablon, E., Ottevaere, I., Durantel, D., Vieux, C., Trepo, C., Zoulim, F., 2004. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy. J. Med. Virol. 74, 41–53.

Wang, X.P., Goodwin, L., Kahn, P., Gawel, C., Cunha, C.B., Laser, B., Sahn, B., Kaplan, M.H., 2006. Influence of increased CD4 cell counts on the genetic variability of hepatitis C virus in patients co-infected with human immunodeficiency virus I. J. Biomol. Tech. 17, 228–239.

Yahoo, N., Sabahi, F., Shahzamani, K., Malboobi, M.A., Jabbari, H., Sharifi, H., Mousavi-Fard, S.H., Merat, S., 2011. Mutations in the E2 and NS5A regions in patients infected with hepatitis C virus genotype 1a and their correlation with response to treatment. J. Med. Virol. 83, 1332–1337.

Zeuzem, S., Asselah, T., Angus, P., Zarski, J.P., Larrey, D., Mullhaupt, B., Gane, E., Schuchmann, M., Lohse, A., Pol, S., Bronowicki, J.P., Roberts, S., Arasteh, K., Zoulim, F., Heim, M., Stern, J.O., Kukolj, G., Nehmiz, G., Haefner, C., Boecher, W.O., 2011. Efficacy of the Protease Inhibitor BI 201335, Polymerase Inhibitor BI 207127, and Ribavirin in Patients with Chronic HCV infection. Gastroenterology 141, 2047–2055.

Zeuzem, S., Lee, J.H., Roth, W.K., 1997. Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa. Hepatology 25, 740–744.

Zignego, A.L., De Carli, M., Monti, M., Careccia, G., La Villa, G., Giannini, C., D'Elios, M.M., Del Prete, G., Gentilini, P., 1995. Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. J. Med. Virol. 47, 58–64.