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No influence of antiretroviral therapy on the mutation rate of the HCV NS5B polymerase in HIV/HCV-coinfected patients

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ABSTRACT

Objectives: To assess the impact of antiretroviral treatment (ART), including nucleoside analogues retrotranscriptase inhibitors (NRTIs), on the mutation rate of hepatitis C virus (HCV) NS5B polymerase and on the ratio of substitution at synonymous and nonsynonymous sites (dN/dS) this polymerase in HIV/HCV-coinfected patients.

Patients and methods: Sixty-one patients on defined ART were included in this study. The NS5B polymerase of HCV was sequenced at baseline and after at least two years of ART. The mutation rate and the dN/dS were calculated at both times.

Results: The NS5B gene from forty-nine (80.3%) patients including; 19 HCV-1a (38.8%), 13 HCV-1b (26.5%), 8 HCV-3a (16.3%) and 9 HCV-4d (18.4%), could be sequenced. Thirty-two (65.3%) patients received non-nucleoside analogues and 41 (83.7%) received protease inhibitor. The mean estimated substitution rates at baseline and at the end of follow-up were from 1.38 to 3.5×10^{-3} substitution/site/year (s/s/y) and from 1.39 to 3.18×10^{-3} s/s/y, respectively, varying according to HCV genotype. All HCV genotypes at baseline and the end time point had values of dN/dS <1. At the end of follow-up, most of sites experienced negative selection and positive selection occurred only in a few sites.

Conclusion: The mutation rate of NS5B in HIV/HCV-coinfected patients is within the range previously reported in studies in HCV-monoinfected patients. Additionally, the use of ART, including NRTIs, in these patients does not affect neither mutation rate nor the dN/dS of the HCV NS5B protein, suggesting that its use would not generate new resistance mutants to the polymerase inhibitors of HCV.

1. Introduction

Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) coinfection is common (Alter, 2006), and the rates of HCV-associated liver disease progression and liver failure are significantly increased in HCV/HIV-coinfected patients (Macías et al., 2010; Pineda et al., 2007). Fortunately, the advent of highly active antiretroviral therapy has resulted in a decrease in the risk of liver-related death among HIV/HCV-coinfected individuals (Qurishi et al., 2003). However, the use of antiretroviral drugs has been associated with an increase in the diversity and variability of HCV in some studies (Blackard et al., 2004; Cristina et al., 2007; Shuhart et al., 2006; Solmone et al., 2006), but not in others (Moretti et al., 2010; Winters et al., 2010). Furthermore, there is contradictory information about the effects of HIV infection on the HCV evolution (Danta et al., 2008; López-Labrador et al., 2007; Netski et al., 2008; Tanaka et al., 2007). Most studies have provided evidence of decreased genetic diversity in HIV/HCV-coinfected patients with respect to HCV-monoinfected individuals, which could be due to reduced immune selective pressure in HIV/HCV coinfection (Danta et al., 2008; López-Labrador et al., 2007). Nevertheless, the results are also conflicting (Netski et al., 2008; Tanaka et al., 2007).

HCV evolution is mostly determined by the number of viral replication cycles, the frequency of nucleotide miss incorporations and the host-mediated and antiviral selection pressures. Understanding HCV evolution is important since viral diversity could be relevant in the outcome of the infection and liver disease progression (Chayama and Hayes, 2011; Farci et al., 2000; Sheridan et al., 2004) On the other hand, changes on HCV sequences, either synonymous substitutions (dS) or nonsynonymous substitutions (dN), could offer information about sites under selection capable to generate resistant mutants. Not only the mutation rate, but also the relative proportion of these two types of mutations (dN/dS), could be

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different in HIV/HCV-coinfected patients and could provide us with important data about HCV viral adaptability in the scenario of HIV-coinfection and ART. An increase in HCV mutation rate could lead to the generation of mutants resistant to the new direct-acting antivirals (DAA) against HCV. In fact, mutations reducing the inhibitory activity of the drugs against the HCV viral polymerase have been described (Lam et al., 2011a,b; Le Pogam et al., 2008; Margeridon-Thermet and Shafer, 2010; Sarrazin and Zeuzem, 2010). Recently, a study assessed the impact of antiretroviral treatment (ART) on the presence of resistant mutations to DAA in HCV NS5B polymerase in HIV/HCV-coinfected individuals (Plaza et al., 2011). This study, found that ART is not associated with the emergence of such viral variants. However, it remains unknown if the mutation rate in adjacent sites of the NS5B gene, where resistant mutants could be generated, remains constant in patients under ART treatment. In addition, even when it is well-known that the rate of mutations of HCV-monoinfected patients is around $1–5 \times 10^{-3}$ substitutions per site per year (s/s/y) (Ogata et al., 1991; Pybus et al., 2001; Smith et al., 1991; Tanaka et al., 2002), less is known about the HCV mutation rate in HIV/HCV-coinfected patients and the effect of ART on HCV mutation rate.

Thus, the aims of this study were to assess the mutation rate and the dN/dS ratio of the HCV NS5B region in HIV/HCV-coinfected patients before starting and after a prolonged period of time on ART.

2. Patients and methods

2.1. Design and study population

This was a retrospective longitudinal study that included HIV/HCV-coinfected patients seen between November 1992 and July 2010 at the Infectious Diseases Units of two tertiary care centers in Seville and Madrid who fulfilled the following criteria: (1) ART-naïve at baseline; (2) Started ART that was stable for at least two years including nucleoside reverse transcriptase inhibitors; (3) Never exposed to pegylated interferon plus ribavirin with or without any DAA for treating hepatitis C; (4) Available serum samples collected before beginning ART and after at least two years of follow-up.

2.2. Laboratory determinations

HCV genotype was determined using a RT-PCR hybridization assay (Versant HCV Genotype2.0 LIPA; Siemens, Tarrytown, NY, USA). The HCV NS5B partial region was amplified and sequenced in both senses from the samples collected at baseline and at the end of follow-up as previously described by Plaza et al. (2011) NS5B sequences were subjected to alignment with CLUSTALX v1.83 software (Thompson et al., 1997).

2.3. Molecular evolutionary rate

Bayesian coalescent-based methods were used to estimate the substitution rate. The estimates of the rate of nucleotide s/s/y were obtained by means of the Bayesian Markov Chain Monte Carlo (MCMC) techniques implemented in the BEAST v1.6.1 program (Drummond and Rambaut, 2007). Both strict and relaxed (uncorrelated lognormal and uncorrelated exponential) molecular clocks were enforced. Five demographic models were applied as coalescent priors: constant population size, exponential growth, expansional growth, logistic growth and Bayesian skyline plot.

These analyses were performed using the general time reversible substitution model with gamma-distributed rates across sites and a proportion of sites assumed to be invariable (GTR+G+I). The best-fit model analyzed here was selected with the Akaike Information Criterion (AIC) by using Modeltest Version 3.06 (Posada and Crandall, 1998). The length and number of Markov chain Monte Carlo (MCMC) were chosen so that the effective sample sizes (ESS) were above 100, indicating that the parameter space was sufficiently explored. The convergence of the parameters to a stationary distribution was assessed with the TRACER v1.5 program (Rambaut and Drummond, 2011), and the statistical uncertainties were summarized from the 95% highest probability density (HPD) intervals. Model comparisons were performed by a Bayes Factor (BF) analysis (Suchard et al., 2001).

2.4. dN/dS ratio and selection

To determine whether positive selection played a substantial role in the generation of mutations associated with drug resistance and the evolution in our samples, we computed the dN/dS ratio of nucleotide substitutions per site for every HCV genotype and time point analyzed in this study. Differences between ratios of dN/dS substitutions were calculated and codons under positive or negative selection were detected via the random effects likelihood (REL) methods as implemented in the Datamonkey program (Kosakovsky Pond and Frost, 2005). *P* values of 0.05 were considered as cutoff for statistical significance in REL method.

Frequencies were compared using the chi-square test or the Fisher's test. The Student's *t*-test and the Mann–Whitney U were used for comparing continuous variables between two groups. The statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM SPSS Inc, Chicago, IL, USA).

2.5. Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the participating hospitals.

3. Results

A total of 61 HIV/HCV-coinfected individuals fulfilled the inclusion criteria. Twenty-eight patients (60.9%) were male, the median [interquartile range (IQR)] age was 46 (40–49) years and the median (IQR) time on ART was 49.6 (30.4–86.5) months. Thirty-two patients (65.3%) received non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 41 (83.7%) received protease inhibitors (PIs). Table 1 summarizes the frequency of antiretroviral drugs

Table 1
Distribution and frequency of antiretrovirals drugs on the study population.

Drug class	Antiretroviral drug	Number of patients (%)
NRTI	Tenofovir	37 (75.5)
	Emtricitabine	26 (53)
	Lamivudine	41 (83.7)
	Zidovudine	28 (57.1)
	Stavudine	25 (51)
	Didanosine	27 (55.1)
	Abacavir	29 (59.2)
NNRTI	Efavirenz	24 (48.9)
	Nevirapine	19 (38.8)
PI	Saquinavir	8 (16.3)
	Lopinavir	25 (51)
	Atazanavir	19 (38.8)
	Nelfinavir	11 (22.4)
	Indinavir	21 (42.8)

NRTI: nucleoside reverse transcriptase inhibitors.
NNRTI: non-nucleoside reverse transcriptase inhibitors.
PI: Protease inhibitors.

Table 2
Estimates of the substitution rate for the NS5B gene by Bayesian coalescent.

No. of NS5B Sequences	Time point and HCV genotype	Mutation rate s/s/y (HPD 95%)	ESS
19	Baseline 1a	1.38×10^{-3} (1.64×10^{-4}/2.67×10^{-3})	194
	End of follow-up 1a	1.39×10^{-3} (2.80×10^{-4}/2.24×10^{-3})	200
13	Baseline 1b	2.72×10^{-3} (7.04×10^{-4}/4.4×10^{-3})	230
	End of follow-up 1b	2.10×10^{-3} (4.82×10^{-4}/3.49×10^{-3})	279
8	Baseline 3a	2.29×10^{-3} (5.20×10^{-4}/2.15 x10^{-3})	432
	End of follow-up 3a	2.04×10^{-3} (2.6×10^{-4}/3.64×10^{-3})	316
9	Baseline 4d	3.50×10^{-3} (2.0×10^{-4}/6.73×10^{-3})	168
	End of follow-up 4d	3.18×10^{-3} (8.07×10^{-4}/5.49×10^{-3})	257

HPD 95%: highest probability density 95%.
ESS: effective sample size.
s/s/y: substitution/site/year.

prescribed to the patients. The median (IQR) plasma HIV–RNA level at baseline was 4.4 (3.6–4.8) log copies/ml vs. 1.5 (1.5–1.7) log copies/ml at the end of follow-up ($p < 0.001$). The baseline CD4 count (IQR) was 309 cells/mL (173–427) and 360 cells/mL (188–590) at the end of follow-up (p 0.022). NS5B partial sequences could be obtained at both time points in 49 (80.3%) patients. In these patients, the distribution of HCV genotypes was as follows: 19 HCV-1a (38.8%), 13 HCV-1b (26.5%), 8 HCV-3a (16.3%) and 9 HCV-4d (18.4%).

3.1. Molecular evolutionary rate

In all genotypes, the BF analysis favored the relaxed uncorrelated exponential molecular clock and the exponential population size over the other models for the partial NS5B analyzed herein. Lengths of MCMC chains of 50 millions for HCV genotypes 1a and 1b and 500 millions for genotypes 3a and 4d were needed to reach values of ESS above 100. With this model, the mean estimated substitution rates for HCV-1a were 1.38×10^{-3} (HPD95% = 1.64×10^{-4}; 2.67×10^{-3}), for HCV-1b 2.72×10^{-3} (HPD95% = 7.04×10^{-4}; 4.4×10^{-3}) for HCV-3a 2.29×10^{-3} (HPD95% = 5.2×10^{-4}; 2.15×10^{-3}), and HCV-4d 3.5×10^{-3} s/s/y (HPD95% = 2.0×10^{-4}; $6{,}73 \times 10^{-3}$). Baseline NS5B polymerase sequences exhibited very similar mutation rates to that observed in specimens collected after more than two years of exposure to ART. Table 2 summarizes the mutation rates for each HCV genotype, before and after ART.

3.2. dN/dS ratio and selection

All genotypes at baseline and at the end of follow-up had values of dN/dS <1 with most of sites experimented negative or purifying selection and just a few sites with positive or adaptive selection (Table 3). Thus, the number of positive selection sites for each genotype and time was: HCV-1a end of follow-up, two out of 236 analyzed codons (0.85%); HCV-1b baseline, 2 out of 160 analyzed codons (1.25%); HCV-1b end of follow-up, 1 out of 160 analyzed codons (0.62%) and HCV-4d baseline and end of follow-up, three out of 187 analyzed codons (1.6%). The dN/dS ratios for both times (baseline and end of follow-up) for each HCV genotype are shown in Table 3.

Table 3
dN/dS ratio and selected sites.

No. of NS5B sequences	Time point and HCV genotype	dN/dS	No. of (−) Sites	No. of (+) site (NS5B position)*
19	Baseline 1a	0.059	99	0
	End of follow-up 1a	0.059	43	2 (95–203)
13	Baseline 1b	0.123	26	2 (207–215)
	End of follow-up 1b	0.098	123	1 (215)
8	Baseline 3a	0.147	146	0
	End of follow-up 3a	0.070	146	0
9	Baseline 4d	0.129	17	3 (58, 128, 243)
	End of follow-up 4d	0.117	13	3 (58, 128, 186)

* In brackets are represented the number of aminoacids positions starting at the first NS5B aminoacid.

4. Discussion

This is the first study, to our knowledge, which has analyzed the mutation rate of HCV in HIV/HCV-coinfected patients. We have found that HCV NS5B variability and diversity is not affected by ART. No differences in variability or diversity of NS5B region were found between baseline, before starting ART, and the end of follow-up, after a prolonged period of time on ART. The dN/dS ratios were <1 and with a high number of sites with negative selection, indicating strong purifying selection pressure for this region.

The mean estimated substitution rates for NS5B in HIV/HCV-coinfected patients were between 1.38 and 3.5×10^{-3} s/s/y, depending on the HCV genotype. These values were within the range of those previously reported for HCV-monoinfected patients (Ogata et al., 1991; Pybus et al., 2001; Smith et al., 1991; Tanaka et al., 2002). It is important to note that we have avoided the methodological limitations due to the use of a strict molecular clock, by employing a relaxed molecular clock that does not assume a constant rate across lineages. In this way, these results are important because the lack of information about the mutation rate of HCV in HIV/HCV-coinfected patients. Furthermore, previous studies have typically evaluated HCV mutations rates using a single HCV genotype or subtype, generally HCV-1a or 1b. On the contrary, in this study the mutation rates of several subtypes (1a, 1b, 3a and 4d) have been calculated, which could represent a first step for future evolutive analyses in unexplored subtypes.

It has been proven that some viral polymerase inhibitors of specific virus could induce cross-variations in viral polymerases of other virus, as demonstrated the emergence of HIV-resistant strains in HIV-infected patients using entecavir, an antiviral for treating hepatitis B (McMahon et al., 2007). Therefore, the same mechanism may act in HIV/HCV-coinfected patients increasing the rate of mutations and generating, in this way, mutations associated with resistance to DAAs. On the other hand, it has been shown that after ART, the heterogeneity of HCV quasispecies significantly changes in HIV/HCV-coinfected patients (Blackard et al., 2004; Cristina et al., 2007; Shuhart et al., 2006; Solmone et al., 2006). Subsequently, viral variations are probably driven by changes in immune pressures induced by ART. These findings suggest that in patients undergoing ART, the HCV variability is affected and depends on the exposition of the analyzed region to the immune system and the methodologies used. However, in this study, no differences were observed in mutation rates for each genotype of HCV NS5B when comparing specimens collected before the beginning of ART and after an average time of 4.3 years

of exposure to antiretroviral drugs. These results support that ART, and specifically NRTIs, do not increase the rate of mutation of NS5B polymerase.

Moreover, the median dN/dS ratio values for all analyzed NS5B were less than 0.15, which is indicative of a relatively strong purifying selection against nonsynonymous changes. Probably, this effect is due to the presence of constrains in NS5B where a nonsynonymous change may have a deleterious effect on viral replication, as recently observed by Blackard et al. in *in vivo* assays (Blackard et al., 2010). Therefore, these results indicate that purifying selection acts to preserve the function of the viral protein despite the high mutation rate inherent to HCV replication. In addition, none of the detected sites with positive selection has been previously described as positions with mutations associated with drug resistance.

It has been described that drug-resistant variants emerge at frequencies of 5–20% of the total virus population as early as at the second day after treatment with DAA (Rong et al., 2010). Here, we observed that such emergence of drug resistance has not appeared as result of ART, at least, in the master sequence. Hence, we hypothesize that, even being very likely that all mutations are already present before starting any treatment, the pressure exerted by ART is not strong enough to select for the presence of preexistent mutations in the master sequence, suggesting that these are not positively selected. Additionally, among the samples from the entire study period we found evidence for positive selection only at a few sites and, to our knowledge, none of these sites were previously described as related with resistance to DAA. However, specific functional assays should be carried out to assess more precisely how the sites with positive selection detected in this study impact on the resistance to DAA. The number of variation observed at sites experiencing adaptive or positive evolution suggests that baseline and end of follow-up samples do not differ substantially and that mutations related with resistance to DAAs do not occur regularly in NS5B over the course of ART. As a consequence, this further analysis confirms, in a much higher number of nucleotide positions, our previous observation that the use of antiretroviral drugs does not increase the rate of primary point drug resistance mutations to HCV NS5B polymerase inhibitors currently under development (Plaza et al., 2011).

Our study has some limitations. The study population was exposed to the majority of antiretroviral agents used in current clinical practice, but not all. Thus, we cannot exclude that some NRTIs, poorly represented in our population, as abacavir, might have some effects on the mutation rate of the NS5B analyzed region. However, a reasonable representation of many other NRTIs was tested in our study population and we can assume that effect of NRTIs on HCV variability was (Plaza et al., 2011). Also, we have not analyzed the NS3/4 region of HCV, where new DAA like Boceprevir or Telaprevir are directed. However, several studies had observed that NS3/4 protease diversity previously and after antiretroviral therapy, including protease inhibitors, was not significantly different (Trimoulet et al., 2011; Winters et al., 2010). Furthermore, no emergence of new resistant mutation related to these DAAs have been observed (Trimoulet et al., 2011; Winters et al., 2010)" in these patients. On the other hand, we had analyzed a small group of patients, especially those infected by genotype 3 and 4. Nevertheless, as the substitution rate of HCV is high enough to produce a substantial number of substitutions in the analyzed time, the convergence was reached for each genotype. Lastly, conventional sequencing method was used to determine the sequences in this study, thus, only the master sequences were analyzed but not the quasispecies. The master sequences are present at a frequency higher than 10–15% of the total of quasiespecies infecting a patient. Nonetheless, the analysis of quasispecies may be considered more relevant in regions with a higher rate of mutation like hipervariable regions, but not in more conserved regions as NS5B, 5'UTR, 3'UTR or Core. Our methodology gives a more global view favoring the examination of the evolution at two time points and allowing the detection of the most representative sequence, i.e. the virus with the highest fitness and frequencies. Additionally, resistant mutations need to be present at a certain frequency (15–20%) to have a considerable effect on viral phenotype (Verbinnen et al., 2010).

5. Conclusion

In summary, the mutation rate and the ratio of dN/dS in the HCV NS5B protein do not appear to be influenced by the use of ART in HIV/HCV-coinfected patients. Consequently, the results presented here suggest that the ART does not increase the mutation rate of NS5B and thus will not enhance the emergence of resistance-associated variants to HCV polymerase inhibitors. As a result, previous exposure to NRTI-based ART is unlikely to have a negative influence on the response to DAA in HIV/HCV-coinfected patients.

Conflict of interest

All authors have nothing to declare.

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