Journal of Clinical Virology 74 (2016) 19–25

Contents lists available at ScienceDirect

Journal of Clinical Virology

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

Duplication of the V3 domain in hepatitis C virus (1b) NS5A protein: Clonal analysis and physicochemical properties related to hepatocellular carcinoma occurrence

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ARTICLE INFO

Article history:
Received 17 April 2015
Received in revised form 28 October 2015
Accepted 6 November 2015

Keywords:
Hepatitis C virus
NS5A-V3 domain
Hepatocellular carcinoma
Duplication
Genetic variability

ABSTRACT

Background: Hepatitis C virus non-structural protein 5A is known to play a role in development of hepatocellular carcinoma (HCC) via interactions with host cell pathways.
Objectives: Hepatitis C virus genotype 1b strains presenting a wide insertion of 31 amino acids in the non-structural protein 5A V3 domain (V3 DI) were studied to determine whether this V3-like additional domain (V3 DII) was associated with HCC occurrence.
Study design: Seventy-four patients' sera were screened for V3 DII presence regarding clinical status.
Results: Three strains with duplicated V3 were detected among patients with progression to HCC ($n = 28$), two strains among patients with liver cirrhosis (Ci, $n = 27$) and none among patients with chronic hepatitis (Chr, $n = 19$). Phylogenetic trees built from V3 DI and V3 DII sequences indicated that the latter clustered separately. In between-group clonal analysis, V3 DII sequences from the HCC group were found to be more distant from HCV-J than V3 DI sequences ($p < 0.0001$). Between-group comparisons showed significant differences in genetic distances from HCV-J, in HCC V3 DI and HCC V3 DII compared to Ci V3 DI and Ci V3 DII sequences ($p < 0.0001$). HCC V3 DII domain and its junction with V3 DI exhibited higher Shannon entropy values and enrichment in disorder-promoting residues.
Conclusions: Taken together, our results suggest that V3 DII evolution may differ in strains associated with HCC occurrence. The presence of an intrinsically "disordered" V3 duplicate may alter the NS5A protein network. Further investigations are necessary to elucidate the potential impact of V3 duplication in the context of carcinogenesis.

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http://dx.doi.org/10.1016/j.jcv.2015.11.011
1386-6532/

1. Background

According to WHO, global hepatitis C virus (HCV) infection prevalence is estimated at 3% of the world population, representing 170 million people. Ten to thirty percent of the latter will become cirrhotic, with a 3–8% per year risk of hepatocellular carcinoma (HCC) development [1]. In developed countries (the USA, Europe and Japan), about 60% of HCC cases are attributed to chronic hepatitis C infection [2]. A recent meta-analysis of published studies evaluating the impact of HCV genotypes on HCC risk revealed a significant association between genotype 1b and higher risk of HCC occurrence [3].

Little is known about the mechanisms by which HCC develops in HCV-related cirrhosis. Four HCV proteins are known to play an important role in hepatic oncogenesis: the core protein [4,5] and the non-structural proteins NS3 [6,7], NS5A [5] and NS5B [8]. NS5A is a promising new target for anti-HCV therapy [9–11] and a host cell growth and apoptosis modulator via interactions with p21, p53 and cyclins [12,13]. NS5A is a large (56–58 kDa, 447 amino acids) RNA binding hydrophilic phosphoprotein organized into three domains (Domains I, II and III) separated by low-complexity sequences [14–17]. NS5A carboxy terminal domain III (356–447) is characterized by a high degree of genetic flexibility. The function of NS5A domain III in the viral replication cycle is still unknown. The NS5A C-terminus can tolerate large heterologous sequence insertions without impairing HCV replication [18,37]. On the other hand, domain III of NS5A may play a key role in hepatitis C virus infectious particle assembly, modulating virion production [14,17].

2. Objectives

In previous French study on HCV genotype 1b NS5A ISDR and V3 domain mutations prior to IFN therapy, Veillon et al. detected strains harboring a never-before-described wide insertion in V3, mimicking a duplication of this domain [19]. A multicenter study was then conducted in France and showed a prevalence of 3.05% for HCV 1b strains with duplicated NS5A V3 domain that may correlate with severity of the liver disease, i.e., fibrosis level and HCC occurrence (Le Guillou-Guillemette et al., twin article).

The aim of the present study was to describe V3 (V3 DI) and V3-like (V3 DII) domain genetic variability, to analyze selective pressure acting on the two paralogs and to estimate physicochemical profiles of this duplication, while comparing strains associated with liver cirrhosis (Ci) or with HCC.

3. Study design

3.1. Patients

Patients' sera were collected from the French Biological Resources Center of Jean Verdier Hospital (Bondy, France). Written consent was obtained for each patient. Seventy-four women with HCV-1b genotype-related liver disease were prospectively recruited according criteria previously reported [6]. Patients were classified into three groups according to the different degrees of liver disease severity at time of serum collection: the Chr group included 19 patients with chronic hepatitis (F1–F2, METAVIR classification), the Ci group included 27 patients with liver cirrhosis (F4) without subsequent development of HCC, and the HCC group, 28 patients with liver cirrhosis who subsequently developed HCC during follow-up. Patients were contaminated in Europe, in most cases via blood transfusion, and were matched for sex, to avoid additional risk factor for HCC development, as male gender.

3.2. HCV-RNA quantification and HCV genotyping

Quantitative detection of HCV-RNA was performed using the RT-PCR-based method (Roche Diagnostics Cobas Amplicor HCV Monitor test v. 2.0, Meylan, France), with a cutoff level at 600 IU mL^{-1}. Genotyping of HCV was carried out by 5′ non-coding region sequencing using the Trugene HCV method (TRUGENE 5′NC, Bayer HealthCare LLC, Berkeley, CA). In case of undetermined subtype, the NS5B region was sequenced to confirm genotype 1b according to French ANRS consensus protocol [20].

3.3. HCV-RNA extraction and NS5A domain V3 amplification by RT-PCR

Total HCV-RNA was extracted from 140 μL of serum using the QIAamp® RNA mini-kit (Qiagen, Courtaboeuf, France). The full-length NS5A gene (6246–7586, HCV-J strain numbering) was first amplified by reverse transcription, combined with an initial PCR using outer primers E1 and E2, previously described by Duverlie et al. [21]. To obtain NS5A V3 domain amplification, a nested PCR was performed with 5 μL of NS5A first PCR product as template and S3/I4 or S31/I4 primers, as previously described by Veillon et al. [22]. Fragment size of nested PCR products (218 or 345 nucleotides using S3/I4 or S31/I4 respectively) was verified by ethidium bromide staining after electrophoresis in a 1% agarose gel (Eurobio, Les Ulis, France). Fragments longer than expected were likely to carry the duplicated V3 domain and were studied.

3.4. NS5A V3 cloning and DNA sequencing

Amplified NS5A V3 domains likely to be duplicated were purified (High Pure PCR Product Purification kit; Roche Molecular Biochemicals, Meylan, France) and ligated into the pCR 2.1™-TOPO vector (TOPO TA Cloning Kit; Invitrogen) following the manufacturer's instructions. Transformants were grown on LB agar plates containing 50 μg mL^{-1} of ampicillin and selected at random. Plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen, Courtaboeuf, France) and subjected to nested NS5A V3 PCR previously described [22]. Amplicon size was verified by electrophoresis in 1% agarose gel. Plasmids containing an insert in the V3 domain were purified and sequenced with M13 universal and M13 reverse primers by the dideoxy chain termination method on a MegaBace DNA analysis system (Amersham Biosciences, Orsay, France). Amplified DNA products were also directly sequenced in both directions using previously described S and I4 primers, with the Big Dye Terminator sequencing kit (Applied Biosystems, Life Technologies, Courtaboeuf, France) on an ABI PRISM 310 sequencer [22].

3.5. NS5AV3 quasispecies analysis

Major sequence alignment was manually edited using the GeneDoc 2.0.1. editor. Quasispecies sequences were aligned with HCV-J V3 as outgroup using the ClustalW program included in MEGA version 4 software [23]. To compare V3 DI and V3 DII diversity according to clinical outcome, genetic distances were estimated within these two regions using the Kimura two-parameter method implemented in MEGA4 software. We next analyzed sequence complexity, defined as Shannon entropy values, at nucleotide and amino acids level, using the Shannon Entropy Two-Tool available on the HCV LANL database (http://hcv.lanl.gov). The number of synonymous (dS) and non-synonymous (dN) substitutions per site was determined using the Nei-Gobojori method with Jukes–Cantor correction in MEGA4 software. The dN/dS ratio at each codon site was estimated using the SNAP tool (synonymous non-synonymous analysis program) available on the HCV LANL database. A ratio

Table 1
Patient characteristics and presence of V3 duplication according clinical stages of liver disease.

	HCC	Ci	Chr	*p*-value
Number of patients	28	27	19	
Age (mean ± SD) (yr)	71.09 ± 8.6	63.19 ± 11.4	56.07 ± 13.0	$p < 0.0001$
Average duration of HCV infection (yr)	31.78	32.83	27.92	$p < 0.05$
Follow-up time (yr)	6.54 (*z*)	5.94 (*y*)	4.55 (*x*)	$p < 0.05$
Gender	Female	Female	Female	
METAVIR score	F4 (100%)	F4 (100%)	F1 (26.3%) F2 (73.7%)	
HCV-RNA mean (range) (UI/mL)[a]	2.98 [0.1–4.93] × 10^6	2.78 [0.07–8.29] × 10^6	4.10 [0.52–15] × 10^6	NS
ALT mean (range) (x ULN)	2.2 [1–6]	2.5 [1–5.83]	2.2 [1–5]	NS
AFP mean (range) (ng/mL)	64 [2–695]	10 [5–55]	5 [3–10]	$p < 0.05$
Number of patients with V3 duplication	3	2	0	NS

HCC: hepatocellular carcinoma; Ci: liver cirrhosis; Chr: chronic hepatitis; yr: years; HCV: hepatitis C virus.
(*x*): average time interval between sampling date and first HCV-positive serology; (*y*): average time interval between sampling date and cirrhosis diagnosis; (*z*): average time interval between sampling date and HCC occurrence.
ALT: alanine aminotransferase; ULN: upper level of normal range; AFP: alpha-fetoprotein; NS: not significant (p-value > 0.05).
[a] IU/mL (Cobas Amplicor Monitor; Roche Diagnostics, Meylan, France).

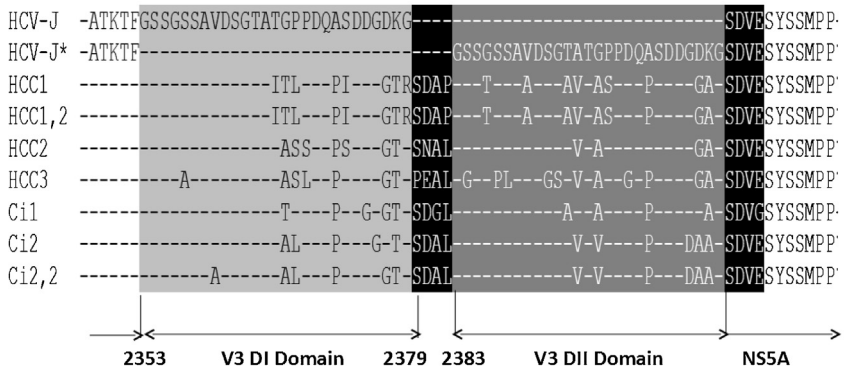

Fig. 1. Alignment of sequences obtained by direct sequencing of NS5A V3 PCR products. The V3 DI domain is shaded in light gray, the additional domain V3 DII in dark gray and the 4-amino-acids junction in black. Dots represent conserved positions in comparison to the HCV-J sequence (top, light gray zone), or to the same sequence artificially reported in the dark gray zone (HCV-J*). HCC: patients with subsequent HCC occurrence; Ci: patients with liver cirrhosis. The alignment was edited using GeneDoc 2.0.1.

Table 2
HCV NS5A V3 DI and V3 DII quasispecies analysis.

Patients and groups	Nb. of sequenced clones	Nb. of different sequences: nt, AA	% id. between V3 DI and V3 DII direct sequences: nt (AA)	Genetic distance[ws]			Genetic distance from HCV-J[bs]		
				V3 DI	V3 DII	*p*-value	V3 DI	V3 DII	*p*-value
HCC ($n = 4$)									
HCC1	27	12, 6	0.7160 (0.6296)	0.0080	0.0089				
HCC1,2	24	21, 18	0.7160 (0.6296)	0.0569	0.0726				
HCC2	19	19, 16	0.8271 (0.8148)	0.0717	0.0773				
HCC3	32	26, 23	0.7901 (0.6296)	0.0292	0.067				
Mean	112		**0.7623** (0.6759)	**0.0415**	**0.0476**	NS	**0.2618**[a]	**0.3414**[b]	$p < 0.0001$
Ci ($n = 3$)									
Ci1	18	10, 8	0.8518 (0.6296)	0.0347	0.0298				
Ci2	30	28, 19	0.8025 (0.7407)	0.0655	0.0998				
Ci2,2	18	10, 7	0.7901 (0.7037)	0.0572	0.0846				
Mean	66		**0.8148** (0.7407)	**0.0525**	**0.0714**	NS	**0.2429**	**0.2353**	NS

HCC: hepatocellular carcinoma; Ci: Liver cirrhosis; V3 DI: V3 domain; V3 DII: V3-like domain.
Id.: identity; nt: nucleotides; AA: amino acids; *p*: *p*-value; NS: non significant.
HCC1, HCC2, HCC3: patients with HCC occurrence; Ci1, Ci2: patients with liver cirrhosis.
HCC1,2 and Ci2,2 serum samples were collected seven years after respectively HCC1 and Ci2.
Mean genetic distance was estimated with the Kimura two-parameter method in MEGA 4 software; ws within-sample; bs between-samples.
Data discussed below are indicated in bold.
[a] Statistically significant difference between the values of HCC V3 DI and Ci V3 DI ($p < 0.0007$).
[b] Statistically significant difference between the values of HCC V3 DII and Ci V3 DII ($p < 0.0001$).

greater than 1 indicated a positive selection pressure, less than 1, a negative selection pressure.

3.6. Hydropathy profiles and physicochemical properties

To delineate the hydrophobic profile of the protein in each clinical group, we used the Kyte–Doolittle hydropathy scale, implemented in DAMBE software [24], with a window size of 6, allowing detection of hydrophilic and possibly antigenic regions. To determine whether quasispecies variations between HCC and Ci groups resulted in physicochemical changes (presence of disorder- or order-promoting amino acids), a graphic comparison of posi-

tional amino acid properties was built using the Two-Sample Logos web-tool [25] available at http://www.twosamplelogo.org/ .

3.7. Statistical analyses

Patient characteristics were compared between groups using analysis of variance or the Wilcoxon–Mann–Whitney test. The difference in V3 duplication prevalence was tested with Fischer's exact test. Differences between groups of sequences were assessed, when appropriate, by Wilcoxon–Mann–Whitney or Student's *t*-tests for quantitative variables. $P < 0.05$ was considered significant.

3.8. Nucleotide sequence GenBank accession numbers

A total of 126 different clonal sequences of 174 nucleotides, coding for NS5A V3 region have been deposited in the GenBank database (accession numbers KF648380–KF648505).

4. Results

4.1. Characteristics of the patients

Seventy-four patients with HCV genotype 1b chronic infection were divided into three clinical groups (HCC, Ci and Chr) and screened for the presence of V3 duplication. Patient general characteristics are presented in Table 1. The average age in each group was statistically different. In HCC and Ci groups, the estimated duration of HCV infection and follow-up time were similar (Table 1). No significant difference was observed in average viral load, nor in average serum alanine aminotransferase levels. The average level of alpha-fetoprotein (AFP) was significantly higher ($p = 0.0389$) among patients who developed HCC.

4.2. Presence and direct sequencing of V3 duplication

Three insertions were detected in the HCC group ($n = 28$, 10.71%), two in the Ci group ($n = 27$, 7.41%) and none in the Chr group ($n = 19$, 0%) but the difference was not statistically significant. The average durations of hepatitis C virus infection in patients with V3 duplication were similar, 45 years for HCC group and 43 years for Ci group. For two patients for whom sequential serum samples were available (one from HCC group, the other from Ci group), V3 duplication was also detected seven years later. In all cases of insertion in the V3 region, an additional domain of 31 amino acids was present, as shown in Fig. 1. The percentages of nucleotide identity between these two domains were high (Table 2).

4.3. Quasispecies composition and phylogeny

Seven HCV-1b isolates were studied for quasispecies analysis. One hundred sixty-seven sequences of 174 nucleotides corresponding to V3 duplicated sequences were obtained for clonal analysis. Phylogenetic trees built from aligned V3 DI and V3 DII clonal nucleotidic sequences showed that V3 DII sequences clustered separately (Fig. 2) [37]. Within these two clades, sequences were grouped according to the quasispecies of each patient. Clustering according to pathology was observed within the V DII clade, where Ci and HCC groups could be considered two sister clades, despite a medium bootstrap resampling value (61). The V3 DI clade showed no specific distribution. When examining an alignment of the 167 clonal sequences of the HCV-1b V3 domain obtained in this study combined with the HCV-J 1b prototype and those previously published (accession numbers: AY808004–AY808042 in the GenBank database), we observed that non-duplicated V3 sequences were aligned with the upstream V3 DI domain of our sequences

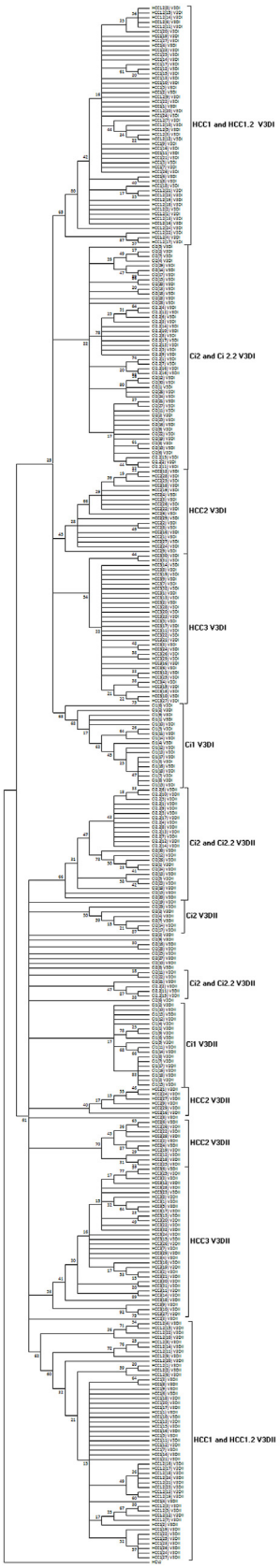

Fig. 2. Phylogenetic tree drawn from 167 clonal sequences of NS5A V3 DI aligned with NS5A V3 DII. Each domain is 27 amino acids (91 nucleotides) in length. The highly variable junction between the two domains has been excluded from phylogenetic analysis. The HCV-J strain was included as outgroup. The tree was built with neighbor-joining method, with a Kimura two-parameter model.

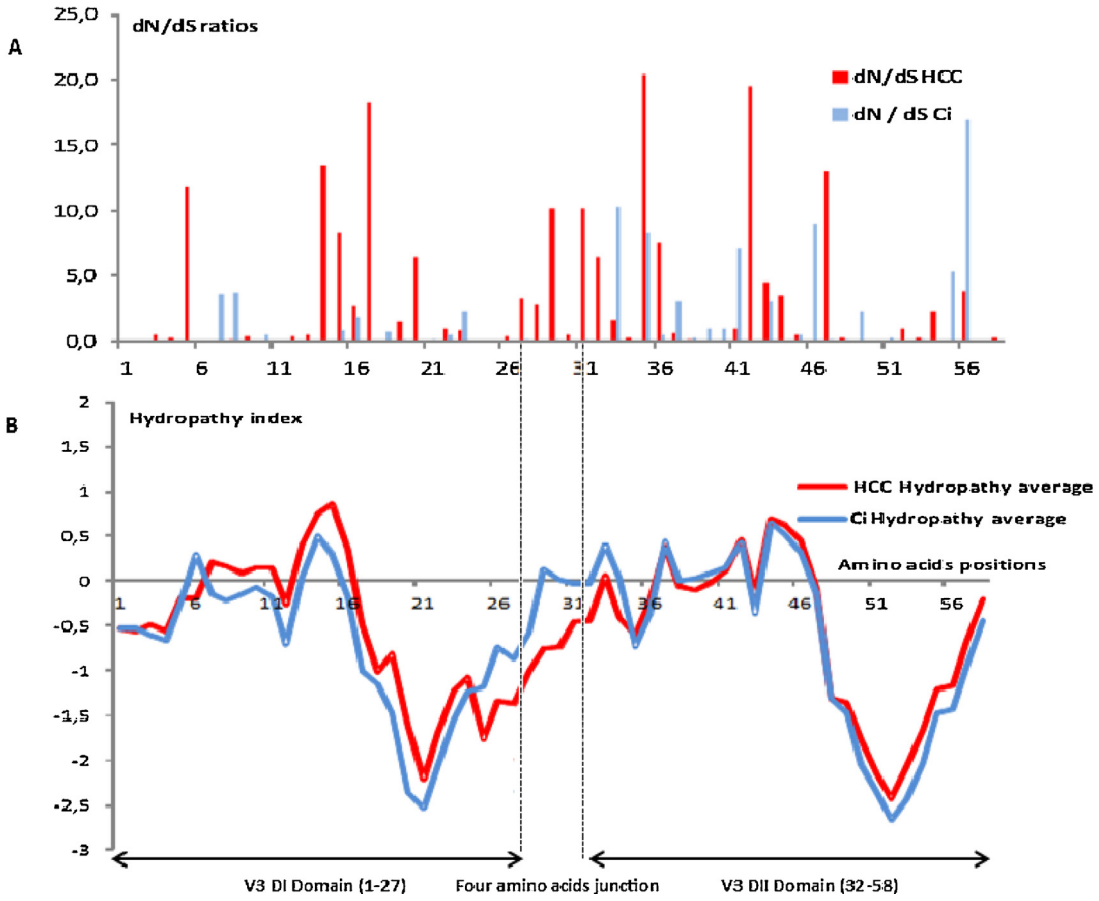

Fig. 3. Graphic representation of variable selective pressures along V3 DI and V3 DII sequences (A) and their hydropathy profiles (B). V3 DI: amino acids 1–27; V3 DII: amino acids 32–58. (A) dN/dS ratios calculated by between-sample analysis using the SNAP tool available on the HCV LANL database. dN/dS values are depicted at the *y*-axis in HCC (HCC occurrence in red) or the Ci group (Ci without evolution to cancer, in blue). Codons are indicated at the *x*-axis (B) Kyte and Doottlittle plots of HCC (in red) and cirrhosis groups (in blue) were calculated using the DAMBE program, with a smoothing window equal to six residues. Each point of the curves is the clone set hydropathy average index. Amino acid positions are indicated at the *x*-axis; hydropathy values are indicated at the *y*-axis.

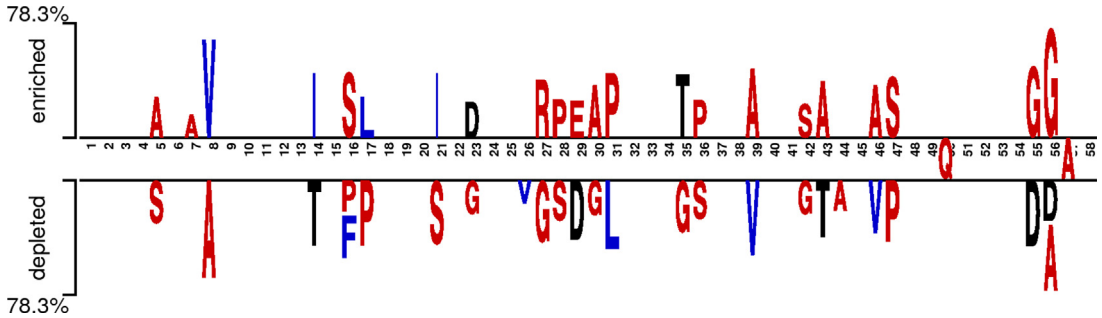

Fig. 4. Two-sample logo of HCC sequence amino acid properties compared to those of Ci. Only the HCC group was represented on the logo, with the upper section displaying statistically significant overrepresented amino acids in the positive set (positive y-axis, p<0.05), the lower section showing statistically significant depleted amino acids (negative y-axis, p<0.05). The height of each amino acid symbol within the logo is proportional to its frequency in the alignment. Disorder-promoting residues: A, R, S, Q, E, G, P (in red); order-promoting residues: N, I, L, V (in blue); disorder-order neutral residues: D, T (in black).

corresponding to codons 2353–2379. Downstream V3 DII (codons 2383–2410) was the additional domain.

4.4. Clonal diversity of V3 domains as related to clinical outcome

By within-sample analysis, genetic distances calculated from V3 DI and V3 DII sequences were similar in each clinical group (Table 2). In between-group comparisons, V3 DII sequences from the HCC group were found to be more distant from HCV-J than V3 DI sequences (Table 2, $p < 0.0001$). In the Ci group, V3 DI and V3 DII sequences were statistically at the same distance from HCV-J. Between-group comparisons showed statistically significant differences in genetic distances from HCV-J, in HCC V3 DI and HCC V3 DII compared to Ci V3 DI and Ci V3 DII sequences (Table 2, $p < 0.0007$ and $p < 0.0001$ respectively). Analyzing positional Shannon's entropy variations in a comparison of HCC group to Ci group, sequences from patients with evolution to cancer revealed higher variability at three amino acids forming junction between V3 DI and V3 DII (positions 28, 29, 30) and along V3 DII (supplementary file, *p*-value from 0.01 to 0.05) [37].

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2015.11.011.

4.5. Physicochemical properties

When comparing HCC and Ci group sequences according to the Kyte–Doolittle scale, the distribution of average hydropathy appeared to be statistically different in most parts of the amino acid sequence, except for residues 1, 2 and 18 (Fig. 3B, *p*-values from 0.0241 to $p < 0.0001$). The junction zone between V3 DI and V3 DII (amino acids 27–31) from patients with evolution to cancer was more hydrophilic. Interestingly, sites characterized by hydropathy variations in the HCC group were under positive selection (Fig. 3A). When comparing their physicochemical properties (Fig. 4), HCC group sequences were enriched in disorder-promoting residues along V3 DII and its junction with V3 DI. At codons 29 and 31, HCC group sequences were depleted of neutral residues (D) and order-promoting residues (L), respectively, but were enriched in disorder-promoting residues, (E) and (P), at six positions (39, 43, 46, 49, 55 and 56).

5. Discussion

Compared to mutation changes, insertions or deletions are quite rare along the HCV genome. While deletions are likely to have damaging effects, insertions that are fixed by natural selection may be neutral or even beneficial to the virus [26]. In this study, strains carrying HCV NS5A V3 duplications were detected in cirrhotic patients before they develop HCC (10.71%) and in cirrhotic patients with no HCC (7.41%), but none in the Chr group. The difference between groups was statistically non-significant, probably due to the rarity of these inserted strains. However, these findings were coherent with the epidemiological study of Le Guillou-Guillemette et al., that reported a V3 duplication prevalence of 9.38% among patients with HCC and 5.75% among cirrhotic patients.

A few reports have analyzed V3 domain mutations in relation to antiviral therapy outcome, but none investigated V3 variability according to liver disease stage or HCC occurrence [22,27–29]. By clonal analysis, the values for genetic distance we obtained for V3 DI and V3 DII were in the range estimated by the studies mentioned previously [22,28,29]. Among patients with evolution to cancer, the V3 DII domain showed statistically significant higher genetic diversity. Furthermore, the higher Shannon entropy values found in the HCC group were particularly concentrated along V3 DII and its junction with V3, suggesting more pronounced complexity of these regions. Maintenance or loss of viral genetic variability depends on host immune selective pressure. Therefore, estimated duration of HCV infection is important for interpreting selection pressure ratios. It was similar in our HCC and Ci groups.

Some authors used in silico methods to predict NS5A carboxy-terminus secondary structure. According to El Hefnawi et al., [32], the region located at 374–410, overlapping V3, is intrinsically disordered, 100% exposed and of low complexity. Disorder-promoting amino acids are less exchangeable in disordered regions than in ordered ones, indicating a potential functional role, as possibly played by V3 DII in HCC patients. NS5A plays a key role in cellular signaling pathways, the dysfunction of which may lead to carcinogenesis [33]. This protein, engaged in multiple protein–protein interactions, could increase its role in the interaction network by incorporating disordered regions and sequence repeats. Like other oncogenic proteins such as the SV40T antigen, papillomavirus E6, adenovirus E1B and HBx from HBV, NS5A is able to hold tumor suppressor p53 in the cell cytoplasm and to disrupt p53-p21/WAF1 cell cycle pathways. According to Lan et al., [34], the NS5A *C*-terminus (amino acids 2135–2419), including the whole V3 domain, interacts with p53. We suggest that, among strains we have studied with known cancer outcome, V3 duplication, by addition of a disordered flexible V3 DII domain, has as a consequence a modification of its ligand binding spectrum, likely to interact with p53. Another argument suggesting a modification in NS5A V3 conformation is the increase of myristoylation sites due to adjunction of the V3 DII domain (five additional sites). According to Yamasaki et al., [35], covalent myristate binding is not reversible and could modify the protein's hydropathy profile and its interaction network.

Completing the epidemiologic study about the relationship between V3 duplication and HCC occurrence reported in the same issue by Le Guillou-Guillemette et al., we detected, in this work, a different evolution of the additional domain V3 DII, in patients according to liver disease stage, associated with physicochemical properties variations. The significance of this finding remains unclear and must be confirmed by other studies. Whether or not V3 duplication has functional consequences might be ascertained by interactome methods [36].

Competing interests

None declared.

Ethical approval

An information letter was sent to all patients and the study was approved by the Ethics Committee of the University Hospital of Brest (Avis CPP Ouest 6-15 11 2006). The study was also approved and registered by the national commission for information technology and civil liberties (CNIL, 907057-09032007).

Acknowledgements

This work was supported by a grant from the French Research Agency for AIDS and Hepatitis ANRS. We wish to thank Prof. Ferec for the sequencing platform access and Jerry Bram, English medical translator, for reviewing this manuscript.

References

[1] D. Lavanchy, The global burden of hepatitis C, Liver Int. 29 (Suppl. 1) (2009) 74–81 [PubMed: 19207969].
[2] F.X. Bosch, J. Ribes, R. Cleries, M. Diaz, Epidemiology of hepatocellular carcinoma, Clin. Liver Dis. 9 (2005) 191–211, v. [PubMed: 15831268].
[3] S. Raimondi, S. Bruno, M.U. Mondelli, P. Maisonneuve, Hepatitis C virus genotype 1b as a risk factor for hepatocellular carcinoma development: a meta-analysis, J. Hepatol. 50 (2009) 1142–1154 [PubMed: 19395111].
[4] M. Anzola, Hepatocellular carcinoma: role of hepatitis B and hepatitis C viruses proteins in hepatocarcinogenesis, J. Viral Hepat. 11 (2004) 383–393 [PubMed: 15357643].
[5] A. Kasprzak, A. Adamek, Role of hepatitis C virus proteins (C, NS3, NS5A) in hepatic oncogenesis, Hepatol. Res. 38 (2008) 1–26 [PubMed: 17894800].
[6] S. Vallet, S. Gouriou, G. Nkontchou, H. Hotta, M. Vilerio, M.C. Legrand-Quillien, et al., Is hepatitis C virus NS3 protease quasispecies heterogeneity predictive of progression from cirrhosis to hepatocellular carcinoma? J. Viral Hepat. 14 (2007) 96–106 [PubMed: 17244249].
[7] S. Ogata, R.H. Florese, M. Nagano-Fujii, R. Hidajat, L. Deng, Y. Ku, et al., Identification of hepatitis C virus (HCV) subtype 1b strains that are highly, or only weakly, associated with hepatocellular carcinoma on the basis of the secondary structure of an amino-terminal portion of the HCV NS3 protein, J. Clin. Microbiol. 41 (2003) 2835–2841 [PubMed: 12843009].
[8] A. Banerjee, R.B. Ray, R. Ray, Oncogenic potential of hepatitis C virus proteins, Viruses 2 (2010) 2108–2133 [PubMed: 21994721].
[9] G.T. Everson, K.D. Sims, M. Rodriguez-Torres, C. Hézode, E. Lawitz, M. Bourlière, V. Loustaud-Ratti, V. Rustgi, H. Schwartz, H. Tatum, P. Marcellin, S. Pol, P.J. Thuluvath, T. Eley, X. Wang, S.P. Huang, F. McPhee, M. Wind-Rotolo, E. Chung, C. Pasquinelli, D.M. Grasela, D.F. Gardiner, Efficacy of an interferon- and ribavirin-free regimen of daclatasvir, asunaprevir, and BMS-791325 in treatment-naive patients with HCV genotype 1 infection, Gastroenterology 146 (2) (2014) 420–729, http://dx.doi.org/10.1053/j.gastro.2013.10.057, Epub 2013 Oct 30. PMID: 24184132.
[10] M. Gao, R.E. Nettles, M. Belema, L.B. Snyder, V.N. Nguyen, R.A. Fridell, et al., Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect, Nature 465 (2010) 96–100 [PubMed: 20410884].
[11] D.A. Herbst, K.R. Reddy, NS5A inhibitor, daclatasvir, for the treatment of chronic hepatitis C virus infection, Expert Opin. Investig. Drugs 22 (2013) 1337–1346 [PubMed: 23931586].

[12] M. Majumder, A.K. Ghosh, R. Steele, R. Ray, R.B. Ray, Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner, J. Virol. 75 (2001) 1401–1407 [PubMed: 11152513].
[13] A.K. Ghosh, R. Steele, K. Meyer, R. Ray, R.B. Ray, Hepatitis C virus NS5A protein modulates cell cycle regulatory genes and promotes cell growth, J. Gen. Virol. 80 (Pt. 5) (1999) 1179–1183 [PubMed: 10355764].
[14] N. Appel, M. Zayas, S. Miller, J. Krijnse-Locker, T. Schaller, P. Friebe, et al., Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly, PLoS Pathog. 4 (2008) e1000035 [PubMed: 18369481].
[15] T.L. Tellinghuisen, J. Marcotrigiano, A.E. Gorbalenya, C.M. Rice, The NS5A protein of hepatitis C virus is a zinc metalloprotein, J. Biol. Chem. 279 (2004) 48576–48587 [PubMed: 15339921].
[16] T.L. Tellinghuisen, K.L. Foss, J.C. Treadaway, C.M. Rice, Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein, J. Virol. 82 (2008) 1073–1083 [PubMed: 18032500].
[17] T.L. Tellinghuisen, K.L. Foss, J. Treadaway, Regulation of hepatitis C virion production via phosphorylation of the NS5A protein, PLoS Pathog. 4 (2008) e1000032 [PubMed: 18369478].
[18] D. Moradpour, M.J. Evans, R. Gosert, Z. Yuan, H.E. Blum, S.P. Goff, et al., Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes, J. Virol. 78 (2004) 7400–7409 [PubMed: 15220413].
[19] P. Veillon, C. Payan, C. Gaudy, A. Goudeau, F. Lunel, Mutation analysis of ISDR and V3 domains of hepatitis C virus NS5A region before interferon therapy with or without ribavirin, Pathol. Biol. (Paris) 52 (2004) 505–510, PubMed: 15531113].
[20] S. Laperche, F. Lunel, J. Izopet, S. Alain, P. Deny, G. Duverlie, et al., Comparison of hepatitis C virus NS5b and 5′ noncoding gene sequencing methods in a multicenter study, J. Clin. Microbiol. 43 (2005) 733–739 [PubMed: 15695672].
[21] G. Duverlie, H. Khorsi, S. Castelain, O. Jaillon, J. Izopet, F. Lunel, et al., Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity, J. Gen. Virol. 79 (Pt 6) (1998) 1373–1381, PubMed: 9634077].
[22] P. Veillon, C. Payan, H. Le Guillou-Guillemette, C. Gaudy, F. Lunel, Quasispecies evolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon-ribavirin therapy, World J. Gastroenterol. 13 (2007) 1195–1203 [PubMed: 17451199].
[23] S. Kumar, M. Nei, J. Dudley, K. Tamura, MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences, Brief Bioinform. 9 (2008) 299–306 [PubMed: 18417537].
[24] X. Xia, Z. Xie, DAMBE: software package for data analysis in molecular biology and evolution, J. Hered. 92 (2001) 371–373 [PubMed: 11535656].
[25] V. Vacic, L.M. Iakoucheva, P. Radivojac, Two sample logo: a graphical representation of the differences between two sets of sequence alignments, Bioinformatics 22 (2006) 1536–1537 [PubMed: 16632492].
[26] M. Torres-Puente, J.M. Cuevas, N. Jimenez-Hernandez, M.A. Bracho, I. Garcia-Robles, F. Carnicer, et al., Contribution of insertions and deletions to the variability of hepatitis C virus populations, J. Gen. Virol. 88 (2007) 2198–2203 [PubMed: 17622623].
[27] J. Nousbaum, S.J. Polyak, S.C. Ray, D.G. Sullivan, A.M. Larson, R.L. Carithers Jr., et al., Prospective characterization of full-length hepatitis C virus NS5A quasispecies during induction and combination antiviral therapy, J. Virol. 74 (2000) 9028–9038 [PubMed: 10982347].
[28] A.C. Jardim, L.H. Yamasaki, A.T. de Queiroz, C. Bittar, J.R. Pinho, C.M. Carareto, et al., Quasispecies of hepatitis C virus genotype 1 and treatment outcome with peginterferon and ribavirin, Infect. Genet. Evol. 9 (2009) 689–698 [PubMed: 19063998].
[29] M.M. ElHefnawi, S. Zada, I.A. El-Azab, Prediction of prognostic biomarkers for interferon-based therapy to hepatitis C virus patients: a meta-analysis of the NS5A protein in subtypes 1a, 1b, and 3a, Virol. J. 7 (2010) 130 [PubMed: 20550652].
[32] M.M. El Hefnawi, W.H. El Behaidy, A.A. Youssif, A.Z. Ghalwash, L.A. El Housseiny, S. Zada, Natural genetic engineering of hepatitis C virus NS5A for immune system counterattack, Ann. N. Y. Acad. Sci. 1178 (2009) 173–185 [PubMed: 19845637].
[33] M. Fromer, J.M. Shifman, Tradeoff between stability and multispecificity in the design of promiscuous proteins, PLoS Comput. Biol. 5 (2009) e1000627 [PubMed: 20041208].
[34] K.H. Lan, M.L. Sheu, S.J. Hwang, S.H. Yen, S.Y. Chen, J.C. Wu, et al., HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis, Oncogene 21 (2002) 4801–4811 [PubMed: 12101418].
[35] L.H. Yamasaki, H.A. Arcuri, A.C. Jardim, C. Bittar, I.M. de Carvalho-Mello, P. Rahal, New insights regarding HCV-NS5A structure/function and indication of genotypic differences, Virol J. 9 (2012) 14 [PubMed: 22239820].
[36] B. de Chassey, V. Navratil, L. Tafforeau, M.S. Hiet, A. Aublin-Gex, S. Agaugue, et al., Hepatitis C virus infection protein network, Mol. Syst. Biol. 4 (2008) 230 [PubMed: 18985028].
[37] H. Le Guillou-Guillemette, A. Ducancelle, S. Bertrais, C. Lemaire, A. Pivert, P. Veillon, E. Bouthry, S. Alain, V. Thibault, F. Abravanel, A.R. Rosenberg, C. Henquell, E. André-Garnier, O. Petsaris, S. Vallet, J.B. Bour, Y. Baazia, P. Trimoulet, P. André, C. Gaudy-Graffin, D. Bettinger, S. Larrat, A. Signori-Schmuck, H. Saoudin, B. Pozzetto, G. Lagathu, S. Minjolle-Cha, F. Stoll-Keller, J.M. Pawlotsky, J. Izopet, C. Payan, F. Lunel-Fabiani, Identification of a duplicated V3 domain in NS5A associated with cirrhosis and hepatocellular carcinoma in HCV-1b patients, J. Clin. Virol. 69 (2015) 203–209.