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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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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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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