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Short Communication

Contribution of insertions and deletions to the variability of hepatitis C virus populations

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Little is known about the potential effects of insertions and deletions (indels) on the evolutionary dynamics of hepatitis C virus (HCV). In fact, the consequences of indels on antiviral treatment response are a field of investigation completely unexplored. Here, an extensive sequencing project was undertaken by cloning and sequencing serum samples from 25 patients infected with HCV subtype 1a and 48 patients with subtype 1b. For 23 patients, samples obtained after treatment with alpha interferon plus ribavirin were also available. Two genome fragments containing the hypervariable regions in the envelope 2 glycoprotein and the PKR-BD domain in NS5A were sequenced, yielding almost 16 000 sequences. Our results show that insertions are quite rare, but they are often present in biologically relevant domains of the HCV genome. Moreover, their frequency distributions between different time samples reflect the quasispecies dynamics of HCV populations. Deletions seem to be subject to negative selection.

The hepatitis C virus (HCV) establishes persistent infection in more than two-thirds of infected individuals. The prevalence of chronic hepatitis ranges between 1 and 2 % in industrialized countries (Pawlotsky, 2006), affecting more than 170 million people worldwide (Wasley & Alter, 2000; Shepard *et al.*, 2005). In approximately 20 % of infected people, the disease progresses to cirrhosis, liver failure or hepatocellular carcinoma (Afdhal, 2004). Antiviral therapies employing alpha interferon (IFN-α) were started before even knowing the infectious agent causing hepatitis C (Hoofnagle *et al.*, 1986). Later on, the nucleotide analogue ribavirin was added in combination with IFN-α (Brillanti *et al.*, 1994). More recently, the results of combination therapy were improved by pegylation of IFN-α molecules, which enhanced their efficacy (Manns *et al.*, 2001; Fried *et al.*, 2002). However, antiviral therapies involve a significant toxicity and are effective in only about 50 % of cases (Heathcote & Main, 2005; Dienstag & McHutchison, 2006).

HCV is a positive-strand RNA virus in the genus *Hepacivirus* that belongs to the family *Flaviviridae*. The HCV genome consists of approximately 9400 nt in length and encodes a polyprotein of about 3000 aa. This polyprotein is cleaved by both host and viral proteases to generate three structural (core, E1 and E2) and seven non-structural (p7, NS2–NS5B) proteins (reviewed in Lindenbach & Rice, 2005). The most remarkable feature of HCV is its considerable genetic heterogeneity, typical of RNA viruses, leading to populations composed of a spectrum of mutants usually termed quasispecies (Eigen & Biebricher, 1988).

In this study, we have sequenced two regions of the HCV genome. The E1–E2 region consisted of 169 nt from the envelope 1 (E1) glycoprotein C terminus and 303 nt from the envelope 2 (E2) glycoprotein N terminus (from nt 1322 to 1793 in the reference HCV genome sequence, GenBank accession no. AF009606, Kuiken *et al.*, 2006). This region included hypervariable regions HVR1 (Weiner *et al.*, 1991), HVR2 (Kato *et al.*, 1992) and HVR3 (Torres-Puente, 2004; Troesch *et al.*, 2006). The second region corresponded to 743 nt (6742–7484) from the NS5A protein coding portion of the HCV genome and included the PKR-BD and V3 domains. These two genome fragments were chosen because of their biological relevance of the regions included therein. On the one hand, three hypervariable regions are included in the E1–E2 region analysed: HVR1, which seems to be involved in target cell recognition and virus

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Table 1. Summary table indicating separately for each subtype the distribution of responder and non-responder patients showing insertions, deletions or absence of indels

For simplicity, results from the E1–E2 and NS5A regions are pooled together. Information about treatment response for five patients (none of them showing indels) was not available, and consequently these patients have not been included in this table.

Subtype	Responders			Non-responders		
	Insertion	Deletion	No indels	Insertion	Deletion	No indels
1a	2	0	10	0	1	10
1b	0	0	15	9	3	18

attachment (Penin *et al.*, 2001); HVR2, which could be involved in cell surface receptor binding (Yagnik *et al.*, 2000); and HVR3, which could play a role in the process of binding with host cell receptors and virus entry into host cells (Troesch *et al.*, 2006). On the other hand, two remarkable domains are included in the NS5A region: the V3 domain, seemingly involved in responsiveness to interferon (Duverlie *et al.*, 1998; Durante Mangoni *et al.*, 2003), and PKR-BD, which contains the putative interferon sensitivity determining region (ISDR) and seems to be involved in blocking the cellular antiviral response induced by interferon (Gale *et al.*, 1997, 1998).

In this study, we used a cohort of 73 patients infected with genotype 1 HCV, 25 of them infected with subtype 1a and 48 with subtype 1b (see Supplementary Tables S1 and S2 available in JGV Online). Samples were provided by three hospitals from the Comunidad Valenciana, Spain. A sample from each patient was taken before a combined therapy of IFN-α plus ribavirin was started (T0 sample). Additionally, for 23 non-responder patients, we also obtained a sample after 6 (T1 sample) or 12 months of treatment (T2 sample), or both (Supplementary Tables S1 and S2). There were 41 patients that did not respond to treatment and 27 patients that did respond to treatment but this information was not available for five patients. Experimental procedures (RNA extraction, amplification, cloning and sequencing of viral populations) are detailed in Jiménez-Hernández *et al.* (2007). Briefly, after viral RNA extraction, reverse transcription reactions were performed with random hexadeoxynucleotides in order to prevent any bias during reactions because of unspecific oligonucleotides. Primers employed for subsequent PCR were previously reported (see Table 1 in Bracho *et al.*, 2004). Amplified DNA products for each region were purified with High Pure PCR product Purification kit (Roche) and directly cloned into *Eco*RV-digested pBluescript II SK (+) phagemid (Stratagene). Plasmid DNA was purified with High Pure Plasmid Isolation kit (Roche). Cloned products for the E1–E2 region or the NS5A region were sequenced using vector-based primers KS and SK (Stratagene). Sequencing was carried out using ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems) on an ABI 3700 automated sequencer. Sequences were verified and both strands assembled using the Staden package (Staden *et al.*, 2000). Sequence alignments were obtained using CLUSTAL_X v1.81 (Thompson *et al.*, 1997). For the E1–E2 region, we obtained about 100 clones from each patient, yielding a total of 9944 sequences. For the NS5A region, we obtained from 25 to 96 clones per patient and 5870 sequences in total were determined (Supplementary Tables S1 and S2).

A summary of the results is shown in Table 1, where the distribution of insertions and deletions (indels) for responder and non-responder patients is given separately for each viral subtype. In general terms, indels were not detected in most of the patients (61 of 73 patients) and were mainly found in non-responder patients, and more specifically in those with subtype 1b. In particular, eight patients showed insertions in the E1–E2 region, all of them located at two recurrent points (Fig. 1). Additional information for each patient, such as the position of the insertion detected, viral subtype, treatment response, available time samples, number of sequences obtained, amino acids present in each insertion, number of sequences containing the insertion, the relative frequency of the different types of insertion and total frequency is provided in Supplementary Table S3 (available in JGV Online). On the one hand, six patients showed insertions between positions 57 and 58, with an insertion size ranging between 1 and 4 aa (Fig. 1 and Supplementary Table S3). On the other hand, two patients showed insertions between positions 152 and 153, with an insertion size of 2 or 3 aa (Fig. 1 and Supplementary Table S3). In this region, position 57 coincides with the first position of HVR1, whereas positions 152–153 are within HVR2, which is located between positions 147 and 155 of the analysed fragment.

When more than one time point sample was available for a patient with an insertion detected in the T0 sample (patients C20, G06, G16, G22 and G28), sequences containing the insertions were present in all samples. For three of these patients (patients G16, G22 and G28), insertions were present in all sequences from all time point samples. In two cases, the frequency of the insertions varied between T0 and T1 samples. Whereas, patient C29 showed a remarkable increase from 9.1 (T0 sample) to 100 % (T1 sample), patient G06 presented the opposite tendency, decreasing the frequency of the insertion from 25 (T0

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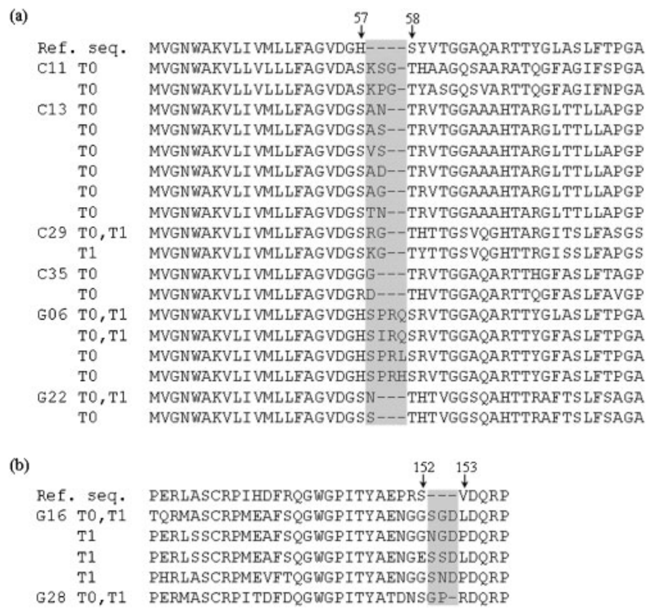

Fig. 1. Amino acid sequence alignment of the patients showing insertions in the E1–E2 region with respect to a reference sequence (Ref. seq.) without insertions. For those patients showing more than one type of insertion, different representative clones are shown, as well as the time point sample where each insertion was found. Grey columns indicate the regions corresponding to the insertions at the positions 57–58 (a), ranging between 1 and 4 aa, and 152–153 (b), ranging between 2 and 3 aa. More detailed information is provided in Supplementary Table S3.

sample) to 9 % (T1 sample). Therefore, in the first patient the insertion got fixed on the viral population, but in the second patient the insertion seemed to tend to extinction. Finally, for patients C11 and C35, where only T0 sample was available, the frequency of insertions was 9.1 and 30 %, respectively.

Only one of the eight patients showing insertions was infected with subtype 1a of HCV. However, no significant differences were detected in the distribution of HCV subtypes between patients with or without insertions in the E1–E2 region (Fisher's exact test, $P=0.250$). Therefore, the apparent excess of patients infected with subtype 1b HCV showing insertions could simply reflect the differential distribution of subtypes in the sample studied.

With regards to the response to antiviral treatment, seven of the eight patients showing insertions were non-responders. Again, no significant differences in the distribution of response or non-response to treatment among patients with or without insertions were observed (Fisher's exact test, $P=0.136$). It is noticeable that all patients infected with subtype 1b HCV were non-responders, whereas the only patient infected with subtype 1a was a responder to the treatment. It is also remarkable that both points of insertion were present in more than one patient, although apparently there is not a common pattern with respect to the nature of the insertion (Supplementary Table S3). It is important to note that these infected patients were epidemiologically unrelated, because previous phylogenetic analyses showed that sequences from each patient grouped in independent clades (data not shown). Therefore, this suggests that a key factor in the dynamics of these

Table 2. Summary of insertions (NS5A region) and deletions (E1–E2 and NS5A regions) detected in the HCV genome

For each patient the position of the insertion/deletion, HCV subtype, treatment response and available time samples, are given. Abbreviations: Seqs, number of analysed sequences for each patient; aas_Ins, amino acids present in the insertion; Seqs_Ins/Del, number of sequences containing a particular insertion/deletion in a given patient; %Tot, percentage of sequences with insertion/deletion in a given patient; Resp, responder patient to therapy; NResp, non-responder patient to therapy.

(a) Patients with insertions in the NS5A region

Patient	Insertion position	Subtype	Treatment response	Time sample	Seqs	aas_Ins	Seqs_Ins	%Tot
A06	6–7	1b	NResp	T0	96	Y	96	100
	94–95	1b	NResp	T0	96	W	2	2.1
C13	94–95	1b	NResp	T0	69	CRR	27	39.1
C33	81–82	1a	Resp	T0	74	L	74	100

(b) Deletions detected at the E1–E2 and NS5A regions

Patient	Region	Deletion position	Deletion size (aa)	Subtype	Treatment response	Time sample	Seqs	Seqs_Del	%Tot
C29	E1–E2	8	1	1b	NResp	T0	100	1	1
A21	NS5A	222	1	1b	NResp	T1	64	1	1.6
A34	NS5A	236–239	4	1a	NResp	T0	29	1	3.5
G22	NS5A	98	1	1b	NResp	T0	36	1	2.8

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mutations is not the type of insertion, but more probably its location.

Three patients showed insertions in the NS5A region, but no recurrence of the insertion points as observed in the E1–E2 region (Table 2a). Patient A06 showed two insertions of 1 aa between positions 6 and 7, and 94 and 95. Patient C33 also showed an insertion in the latter location, but in this case consisting of 3 aa. Finally, patient C13 presented an insertion of 1 aa between positions 81 and 82. Insertion points 81–82 and 94–95 are within the ISDR, which expands from amino acid position 71 to 110 in the analysed fragment. For these patients, only T0 samples were available. The frequency of insertions located at positions 6–7 and 81–82 was 100 %, whereas the insertions at position 94–95 showed a frequency of 39.1 % for patient C13 and of 2.1 % for patient A06.

The number of deletions detected in this study was lower than the number of insertions (Table 1). For the E1–E2 region, patient C29 showed the only deletion identified, which consisted of an amino acid at position 8 detected in its T0 sample (Table 2b). For the NS5A region, three patients presented deletions. In patient A21, a sequence showed an amino acid deleted at position 222 for the T1 sample. Patient A34 presented a sequence with 4 aa deleted at position 236 for the T0 sample. Finally, patient G22 showed a sequence with 1 aa deleted at position 98 for the T0 sample (Table 2b).

Several studies have shown that the quasispecies nature of HCV enables it to evade the immune response (Manzin *et al.*, 1998; Ray *et al.*, 1999). In this sense, the regions under the strongest immune system pressures show the highest degree of genetic variability (Reed & Rice, 2000), as is the case for the HVR1 region. For this region, it has been shown that non-responder patients present an increase in genetic variability (Farci *et al.*, 2000) and that the evolution of HVR1 in the early phases of antiviral treatment can be an indicator of its therapeutic outcome (Farci *et al.*, 2002). Moreover, several studies have observed a correlation between genetic variability of HVR1 and a degree of resistance to antiviral treatments (Polyak *et al.*, 1997) or severity of hepatic injury (Curran *et al.*, 2002; Farci *et al.*, 2006). Very recently, several studies have sought to correlate the outcome of antiviral treatments with the degree of genetic variability in different regions of HCV genome, such as the 5′UTR (Zekri *et al.*, 2007), the p7 membrane protein (Castelain *et al.*, 2007), the NS3 protein (Vallet *et al.*, 2007) or the core region (Akuta *et al.*, 2007).

Because of their low frequency compared with point mutations, only a few studies have described indels in HCV. Therefore, little is known about the consequences of these mutations on viral infective ability or response to antiviral treatment. Different studies have occasionally identified indels in the 5′UTR (Zhang *et al.*, 1999; Revie *et al.*, 2006). Interestingly, indels have been described between positions 384 and 385 for HVR1 (Kato *et al.*, 1992; Abe *et al.*, 1992; Hohne *et al.*, 1994), which corresponds to position 57–58 in our analysed genome fragment, where six of eight insertions in the E1–E2 region were detected. Although insertions seem to appear repetitively in this region, HVR1 size is invariable for all genotypes, apart from genotype 6, which suggests the existence of selective restrictions maintaining an adequate size for this region (Chamberlain *et al.*, 1997).

A few studies have analysed the implications of size variability in HVR1. A statistically significant relationship between the presence of indels at position 384–385 and the development of mixed cryoglobulinemia type 2, a lymphoproliferative disorder associated with chronic HCV infection has been described (Gerotto *et al.*, 2001). According to a structural model predicted for the E2 protein (Yagnik *et al.*, 2000), HVR1, apart from being a highly exposed antigenic site, is located very close to a CD81-binding region, and physical interactions could be established between them. The same happens with HVR2, which is not only a very exposed region but it is also located very close to a region involved in CD81 binding and/or dimerization (Yagnik *et al.*, 2000). In our study, two of the eight insertions detected in the E1–E2 region were located in HVR2. Therefore, changes in HVR1 and HVR2 size could affect their antigenic properties and ability to bind to cellular receptors.

Indels have also been described for the NS5A protein, although at a lower frequency; such is the case of an 8 aa insertion in the ISDR (Enomoto *et al.*, 1996). Size modifications in this protein seem to be genotype specific (Maertens, 1997). Similarly, the analysis of the coding region for 37 HCV whole genomes revealed 12 points with insertions or deletions, many of them strongly associated to a viral subtype (Casino *et al.*, 1999). Therefore, these data suggest a certain influence of indel events at different levels of the HCV evolutionary dynamics, such as differentiation processes between viral subtypes or antiviral treatment response. Again, two of three insertions found in the NS5A region were located within a domain probably involved in immune response evasion, the ISDR, and this suggests that insertions could play a relevant role in this mechanism. In this sense, a recent study has shown that indel analyses permit the identification of regions in the NS5A protein that are not essential for viral genome replication, such as the ISDR or the C-terminal region (Liu *et al.*, 2006).

Some deletions were also found in the analysed patients (Table 2b). Four patients showed deletions, one in the E1–E2 region and three in the NS5A region. For all patients, the deletion was present in a single sequence. This paucity of sequences with deletions could be due to selective pressures against these events because, for compact genomes such as those of RNA viruses, deletions are very likely to be deleterious and to be removed by selection. In contrast, our results show that the frequency of insertions in viral populations can be very high (Supplementary Table S3 and Table 2a), thus suggesting that these kind of mutations promote almost neutral or even beneficial effects on viral

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fitness. At this point, it is important to note that indels are very unlikely to revert and consequently their frequency in viral quasispecies would essentially depend on their effects on fitness.

In summary, our results indicate that the frequency distributions of insertions in the E1–E2 and NS5A regions between different time samples also reflect the naturally occurring quasispecies dynamics of HCV populations. In spite of the relatively low number of cases detected, our results also suggest, for subtype 1b patients, a potential association between the presence of insertions and lack of response to treatment (Table 1), although further studies are necessary to verify this hypothesis.

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