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Rare HVR1-HCV genotype 1b variants in patients with B Non Hodgkin's Lymphoma. Comparison with viral sequences detected in cases of lymphoproliferative disorders and B cell compartmentalisation

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SUMMARY

We compared the E2-HVR1 region in HCV-1b positive B-NHL cases from a multicenter study with sequences from studies related to lymphoproliferative disorders and B cell compartmentalisation. We found rare and unique mutations both in B-NHL isolates and in cases with lymphoproliferative disorders and lymphocyte infection. These rare mutations could have an important effect on HVR1 region and, as a consequence, on the binding of E2 on CD81, with a possible implication for both antigenic stimulation and HCV entry. In conclusion, the HCV predominants circulating in B-NHL cases seem to be associated with clonal selection of rare variants.

KEY WORDS: HCV, Non Hodgkin's lymphoma, Viral variants, Viral variabilità, HVR1

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus belonging to the Flaviviridae family. Although is predominantly a hepatotropic virus, it was found to replicate in mononuclear cells from infected patients (Moldway *et al.,* 1994) and in peripheral blood mononuclear cells (PBMC) of patients with occult HCV infection (Castillo *et al.,* 2005). Virus entry may be mediated by CD81, with involvement of the HCV-E2 protein in a multireceptor complex (Bartosch *et al.,* 2003). Based on *in vitro* studies, it has been proposed that the CD81-mediated proliferation of B cells may be a key factor in the onset of HCV-associated B lymphocyte disorders (Rosa *et al.,* 2005). Although controversial, there is evidence that HCV variants can be compartmentalized in lymphocytes (Ducoulombier *et al.,* 2004; Hsu *et al.,* 1999; Roque Afonso *et al.,* 1999). HCV is associated with a spectrum of extrahepatic manifestations, most of which involve B cells. Several studies have suggested that there exists a link between HCV infection and B-cell non-Hodgkin lymphoma (B-NHL)

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(Mele *et al.,* 2003; Silvestri *et al.,* 1996). However, it remains to be determined whether or not HCV is responsible for the antigenic stimulation that leads to B-cell proliferation and whether it increases the potential of random cell replication errors (Fisher and Fisher, 2004). Several studies have pointed out the importance of the E2 region in lymphoid disorders (Gerotto *et al.,* 2001; Machida *et al.,* 2005; Rosa *et al.,* 2005). In the present study, we molecularly characterised the circulating HCV variants in patients with both B-NHL and HCV infection. We focused our analysis on the E2 hypervariable region 1 (HVR1), which is involved in virus entry and in modulating the E2-CD81 interaction (Roccasecca *et al.,* 2003) and which may thus play an important role in both cell compartmentalization and lymphomagenesis.

The analyses were performed on serum samples previously collected from patients in 10 hospital haematology departments who had participated in an epidemiological study on HCV and B-NHL conducted by our research group (Mele *et al.,* 2003). In the present study, we considered the samples from the 14 anti HCV positive patients with HCV genotype identified as 1b (Innogenetic Line probe assay (Innolipa, Zwijndrecht, Belgium) and for whom serum was sufficient for repeated tests.

We selected patients with HCV genotype 1b, owing to the wide distribution of this genotype and the large availability of databank sequences for comparisons. For the 14 patients, PCR was performed to amplify a fragment of the E2 region (nt 1452-1791) which contains the E2 (HVR1) (nt 1479-1559). HCV-RNA was extracted from 100 ml of serum using the QIAamp UltraSens Virus Kit, Qiagen.

The retrotranscription step was carried out with ImProm-II™ Reverse Transcription System, Promega, using as antisense primer an equimolar mixture of 1b-r1a (nt 2796-2774): 5'ARAACCSCRCCYCCRCAYGATGC3' and 1b-r1b (nt 2792-2769): 5'CCGCGCCYCCGCACGAYGCRGCCA3'. The E2 fragment was amplified by means of nested PCR. The first amplification step was performed using an equimolar mixture of the forward primers 1b-f1a (nt 1393-1414): 5'ARAACCSCRCCYCCRCAYGATGC3', and 1b-f1b: (nt 1410-1432) 5'TAYTATTCYATGGYRGGGAAYTGG3'. The same reverse primers as those used in the retrotranscription step were used. The second amplification step was carried out using an equimolar mixture of the forward primers 1b-F2a (nt 1419-1440): 5'ATGGTRGGGAACTGGGCYAAGGT3' and 1b-F2b (nt 1452-1473): 5'TGCTRCTHTTYGCHGGYGTYGA3' and as reverse primer 1b-R3 (nt 1791-1769): 5'TGCCARCARTAHGGCCTYTGRTC3'.

All positive samples were sequenced using the BigDye Terminator kit (Applied Biosystems, Foster City, CA), on an ABI 373 automatic sequencer. The nucleotide sequences obtained are available in the GenBank database under accession numbers DQ463307-DQ463320.

To perform phylogenetic analyses the E2 sequences from our patients were aligned with the 196 non-redundant HCV-1b E2 sequences from the EU-HCV data bank (http://euhcvdb.ibcp.fr) and manually adjusted by means of the BioEdit program.

The phylogenetic analysis was performed on the E2 fragment (nt 1560 to 1753) using the MEGA package. We compared the deduced amino-acid sequences with a generic (i.e., non-genotype-specific) HVR1 amino-acid repertoire from 1,382 EMBL HCV sequences reported in another study (Penin *et al.,* 2001).

Similarly, we compared 322 HVR1 sequences derived from studies on: HCV infection associated with mixed cryoglobulinemia (MC) type 2 (168 sequences) (Gerotto *et al.,* 2001); HCV replication in CD8+ and CD19+ lymphocytes (55 sequences) (Roque Afonso *et al.,* 1999); ascitic mononuclear cells and PBMC (12 sequences) (Hsu *et al.,* 1999); lymphotropism and compartmentalization of HCV-1b strains (87 sequences from CD14+ and CD19+ cells) (Ducoulombier *et al.,* 2004) and, as control sequences, 196 non-redundant HCV-1b E2 sequences from the EU-HCV data bank. For each sequence group, mutations were identified in comparison with the generic repertoire, and then compared among studies. The residues were classified as rare if present in generic repertoire with frequencies below 10%; unique if not present in repertoire.

The results of the phylogenetic analysis (data not shown) were consistent with those of the Innolipa assay on 5'NCR, confirming that all of the isolates belonged to genotype 1b. The isolates were singularly distributed in different branch-

es within the databank HCV-1b assigned sequences. The comparison of HVR1 nucleotide sequences from our isolates and the HCV-1b prototype showed that the ORF was conserved without deletions or insertions (data not shown), unlike previous observations in MC patients (Gerotto *et al.,* 2001).

Table 1 illustrates the results of the comparisons for the deduced HVR1 amino-acid sequences of the 14 isolates.

We observed that the mean number of rare and unique residues/sequence were double that in control 1b sequences; the other compared group also showed an increase in rare or unique residue frequencies.

We found 21 rare or unique mutations on 14 amino-acid residues that were shared in different measure also by the other compared populations (14 coincident rare or unique mutation on HVR1 residues in MC2 patients; 8 on HVR1 from B-lymphocyte infection; 7 from ascytic mononuclear cells and PBMC infection and 5 from B-cell compartmentalization sequences.

This rare mutation were also present on HVR1 genotype 1b control sequences (13 coincident rare residues), with frequencies generally lower than in B-NHL (range from 2 to 7%). We observed 5 rare residues on B-NHL HVR1 sequences (A1, M5, L14, A22, T25) with frequencies larger than 10%, (rare in comparison with the generic amino-acid repertoire, but not rare in B-NHL population); similarly, also the other groups included in the comparison (excluding the GB-1b control group) showed rare residues with frequencies larger than 10%.

From the comparison of the 5 residue position (1, 4, 10, 18, 20) identified on HVR-1 from B-NHL patients with the other populations included in the analysis, we observed in each group the copresence of rare residues with frequencies >10% in at least 3 out the five positions.

Interestingly, on HVR1 residue positions 1 and 14, we found rare residues with frequencies >10% in all of the compared populations. The identified pattern of rare and unique mutations on HVR1 of predominant sequences of B-NHL HCV cases is shared by other HCV-1b isolates involved in lymphotropic infections and particularly in MC type 2 (a lymphoproliferative disorder that may evolve into B-cell non-Hodgkin lymphoma) (De Re *et al.,* 2000; Pozzato *et al.,* 1994). These patterns are not commonly found in HCV-1b isolates from chronic hepatitis patients and may be characteristic of rare circulating viral clones predominant in lymphotropic variants.

The HVR1 region is usually considered a highly "disordered loop" which may function as a molecular decoy.

The observed pattern of rare or unique residues could be directly or indirectly involved in the increased disorder observed in the HVR1 of the B-NHL associated HCV isolates. We speculate that hypermutation of the HVR1 may have an impact on lymphotropism and B cell activation.

It is believed that continuous antigenic stimulation produced by chronic HCV infection may increase B-cell proliferation and in turn the probability of random genetic mutations, possibly involving immunoglobulin gene rearrangements (Fisher and Fisher, 2004; Gerotto *et al.,* 2001; Machida *et al.,* 2005). In other studies (Rosa *et al.,* 2005; Sansonno *et al.,* 2005), antigenic stimulation in chronic HCV-infected patients was shown to be associated with a strong reduction in the B cell circulating clones.

Moreover, there is evidence that HCV infection can induce clonal cell proliferation in patients with molecular alterations in lymphocytes (i.e., over-expression of Bcl-2 and IgH rearrangements) (Gasparotto *et al.,* 2002). An important observation is the capability of HCV to enter and infect lymphocytes. In our study, we observed that rare HCV variants with a pattern similar to lympotropic strains prevailed in the viral population in B-NHL patients.

HCV replication could be a cofactor in tumour development. To the best of our knowledge, the hypermutations in HCV-infected cells may be the first such case; alternatively, the cognate B cells could simultaneously engage in altering signalling complexes (i.e., B cell receptor and CD19/CD21/CD81 complexes, with CD81 as putative receptor) (Roccasecca *et al.,* 2003). It has been reported that similar changes are able to alter the B cell activation (Quinn *et al.,* 2001).

It may be possible that variations of HVR1 may be directly responsible for reducing the binding of E2 protein to CD81, the putative HCV coreceptor (Roccasecca *et al.,* 2003).

In both cases, the presence of HCV proteins could have an impact on the initiation of growth

TABLE 1 - *Presence of rare and unique amino-acid residues in HVR1 in comparison with generic amino-acid repertoire from 1,382 genotype-unrelated sequences (Penin et al., 2001) for HCV-1b isolates associated with lymphotropic infection and in confirmed genotype 1b non redundant sequences from EU-HCV Databank (control sequences). We reported rare or unique residues only for amino-acid with frequencies at least 1.5%*

HVR1 residues	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*Mean rare+ unique residues/ sequence*
B-NHL; 14 seq.	**a_{29} q_{7}**	-	**m_{7} s_{7}**	**a_{7} c_{7} l_{7}**	**m_{14}**	-	**q_{7}**	**l_{7}**	**i_{7} s_{7}**	-	**s_{7}**	**r_{7} q_{7}**	-	**l_{14} g_{7} c_{7}**	**a_{7} k_{7}**	-	**s_{7}**	**r_{7} a_{7} t_{7}**	-	-	**w_{7} v_{7}**	**a_{21} g_{7}**	-	-	**t_{21} h_{7}**	-	-	**2,8**
MC2 patients, 168 seq. (Gerotto et al., 2001)	**r_{13} a_{11} q_{11} v_{5} l_{4}**	-	**n_{5} v_{4} q_{2}**	-	**m_{13}**	-	**s_{5} q_{3} e_{2}**	**r_{5} e_{5} i_{3}**	**e_{10} d_{10} l_{5} i_{5} h_{5} s_{4}**	-	**l_{5} s_{2}**	**q_{13} i_{2}**	**l_{2}**	**g_{17} n_{10} f_{3}**	**k_{12} l_{5} m_{3} v_{2} a_{2}**		**r_{3}**	**r_{10} a_{7} h_{2}**	-	-	**k_{3}**	**f_{5} q_{5} t_{4} a_{4}**	-	-	**n_{3} t_{2}**	-	**t_{4}**	**2,5**
B lymphocyte infection; 55 seq. (Roque Afonso et al., 1999)	**v_{13} q_{11} y_{9} a_{4}**	-	-	**a_{2}**	**m_{2}**	-	-	**m_{11}**	**e_{11}**	-	**k_{9} c_{4} l_{2}**	-	-	**g_{36} w_{11} m_{6}**	**n_{2}**	-	**s_{9}**	**a_{2}**	**m_{47}**	-	-	-	-	**l_{2}**	**t_{2}**	-	**q_{4} h_{4}**	**2,0**
Ascytic mononuclear cells and PBMC infection; 12 seq. (Hsu et al., 1999)	**k_{25} p_{8}**	**a_{17} p_{8}**	**c_{17} s_{8} p_{8} v_{8} m_{8}**	**a_{25} r_{8}**	**r_{17} g_{17}**	**r_{17}**	-	**g_{17} r_{8}**	**s_{33} e_{17} m_{8} l_{8}**	**r_{8} l_{8}**	**g_{17}**	**r_{17} p_{17} d_{8} h_{8}**	**w_{8} p_{8}**	**g_{25} l_{8} p_{8}**	**w_{8}**	**p_{8} s_{8} t_{8}**	**c_{17} r_{17}**	**a_{33} r_{8}**	**m_{17} v_{8} a_{8} s_{8}**	-	**p_{8}**	**h_{8} i_{8}**	**c_{8} w_{8}**	**r_{8}**	**c_{17}**	**w_{17} v_{8}**	-	**6,7**
B cell compartmentalization; 87 seq. (Ducoulombier et al., 2004)	**a_{24} r_{6}**	**s_{18} r_{17}**	**l_{2}**	**a_{9}**	-	-		**e_{21}**	**d_{26} e_{6}**	**r_{2}**	**w_{15} p_{2}**	**r_{43}**		**f_{12} t_{3}**	**y_{7} l_{6} m_{5} c_{5}**	**q_{6}**	**r_{37}**	**d_{30} a_{7} r_{5}**	**m_{22} s_{5}**	-	**k_{15} q_{2}**	**a_{18} t_{14}**	-		**p_{18}**	**r_{3}**	**d_{47}**	**4,6**
GB-1b 196 seq. (Control)	**q_{7} a_{5} r_{5} v_{3} k_{3}**	-	**v_{2}**	**a_{4}**	**m_{7} i_{6}**	-	-	**e_{4} h_{3} k_{2}**	**s_{7} h_{2}**	**r_{1}**	**f_{4} k_{4} s_{3}**	**h_{3} v_{2}**	**l_{3} i_{2}**	**l_{6} f_{5} n_{4}**	**k_{2}**	-	**m_{2}**	**t_{5} r_{5} a_{4}**	-	-	-	**f_{7} a_{6} v_{5} q_{3}**	-	-	**t_{6}**	-	-	**1,4**

Legend: Unique residues are highlighted. Rare or unique residues present in B-NHL HVR1 and in lymphotropic infection are reported in bold underlined letters. Number at right of residues indicate the amino-acid frequencies.

deregulation which predisposes lymphocyte proliferation, eventually leading to malignancy in the absence of continuous antigenic stimulation. Other observations (Ducoulombier *et al.,* 2004; Gasparotto *et al.,* 2002; Quinn *et al.,* 2001) indicate that in HCV infected cells both cells and viruses may be of clonal origin. We speculate that clonal selection may occur in parallel with the lymphotropic HCV variants which adapt to B-cell clones derived during B-NHL development. The presence of rare or unique mutations for other HCV genotypes should be analysed in future studies.

In conclusion, the HCV predominants circulating in B-NHL-HCV positive patients are rare and probably the result of clonal selection. Similar patterns were observed among HCV variants previously characterised as lymphotropic. The possible role of the rare and unique mutations observed in HCV B-NHL patients is also suggested by their presence in MC type 2 HCV patients. Although this study cannot explain the relationship between B-NHL and HCV infection, we propose studying the predictive value of the presence of several rare HCV variants as markers of lymphoma development.

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