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Short communication

## Emergence of occult minority genotype 2b hepatitis C infection in an HIV-1-co-infected patient treated for genotype 5a HCV infection with 48 weeks of pegylated-interferon- $\alpha$ 2b and ribavirin

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### Abstract

An HIV-1/hepatitis C virus (HCV) co-infected patient with haemophilia received a 48-week course of pegylated interferon- $\alpha$ -2b and ribavirin therapy for genotype 5a HCV infection. Virological response was achieved at week 24. At the end of treatment, HCV RNA in serum was detected and identified to belong to genotype 2b, rather than genotype 5a. A sensitive method for identifying minority HCV genotypes in pre-treatment serum showed genotype 2b HCV carriage prior to treatment. Sequencing the interferon sensitivity-determining region of the HCV NS5A gene obtained from pre-, intra- and post-treatment sera revealed emergence of quasispecies bearing R  $\rightarrow$  K and M  $\rightarrow$  A/T mutations at codons 2222 and 2223, respectively. Occult presence of minority HCV subpopulations and their acquisition of mutations following therapy can result in poor treatment outcome.

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Patients with bleeding disorders given clotting factor concentrates prior to heat treatment are prone to multiple blood-borne virus infections, including dual infections with human immunodeficiency virus (HIV) and hepatitis C virus (HCV). Further, infections with multiple HCV genotypes have been described which may hasten the progression of HIV disease (van Asten and Prins, 2004; Buckton et al., 2006). The success of HCV treatment may also be compromised by the presence of a minority genotype that confers poor response to therapy which subsequently establishes dominance (Schroter et al., 2003). We report an investigation of an HIV/HCV co-infected patient who received 48 week of combined treatment with interferon and ribavirin for presumed mono-infection with genotype 5a HCV, but failed to

achieve a sustained virological response. In this patient, the HCV genotype persisting after treatment was found to be 2b.

The patient was a 40-year-old male with severe haemophilia A, who was co-infected with HIV-1 and HCV. He never had any AIDS defining illness and his CD4 nadir was 125 cells/mm<sup>3</sup>. His HIV infection was effectively controlled with a highly active antiretroviral therapy regimen consisting of tenofovir, lamivudine and boosted lopinavir. His HIV viral load had been less than 50 copies/ml for more than 5 years and CD4 count reached 297 cells/mm<sup>3</sup>. Consensus nucleotide sequencing and phylogenetic analysis of a DNA fragment amplified by reverse transcriptase PCR from the NS5B-encoding region of the HCV genome identified the infecting genotype as 5a. He received 48-week treatment with pegylated interferon- $\alpha$  2b (100  $\mu$ g/week) and ribavirin (400 mg twice daily). HCV RNA clearance was achieved relatively late into treatment, at 24 weeks. Viraemia reappeared

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Table 1  
Outcome of laboratory tests for HCV genotype and serotype

Sample	Time relative to treatment	HCV genotype based on consensus NSSB sequence	HCV serotype	HCV genotype identified by Pyrosequencing
X	Pre-treatment (5 years)	5a	NR	5a + 2b
Y	During treatment (26 weeks)	2b	NR	2b
Z	Post-treatment (5 weeks)	2b	5	2b

NR, not reactive.

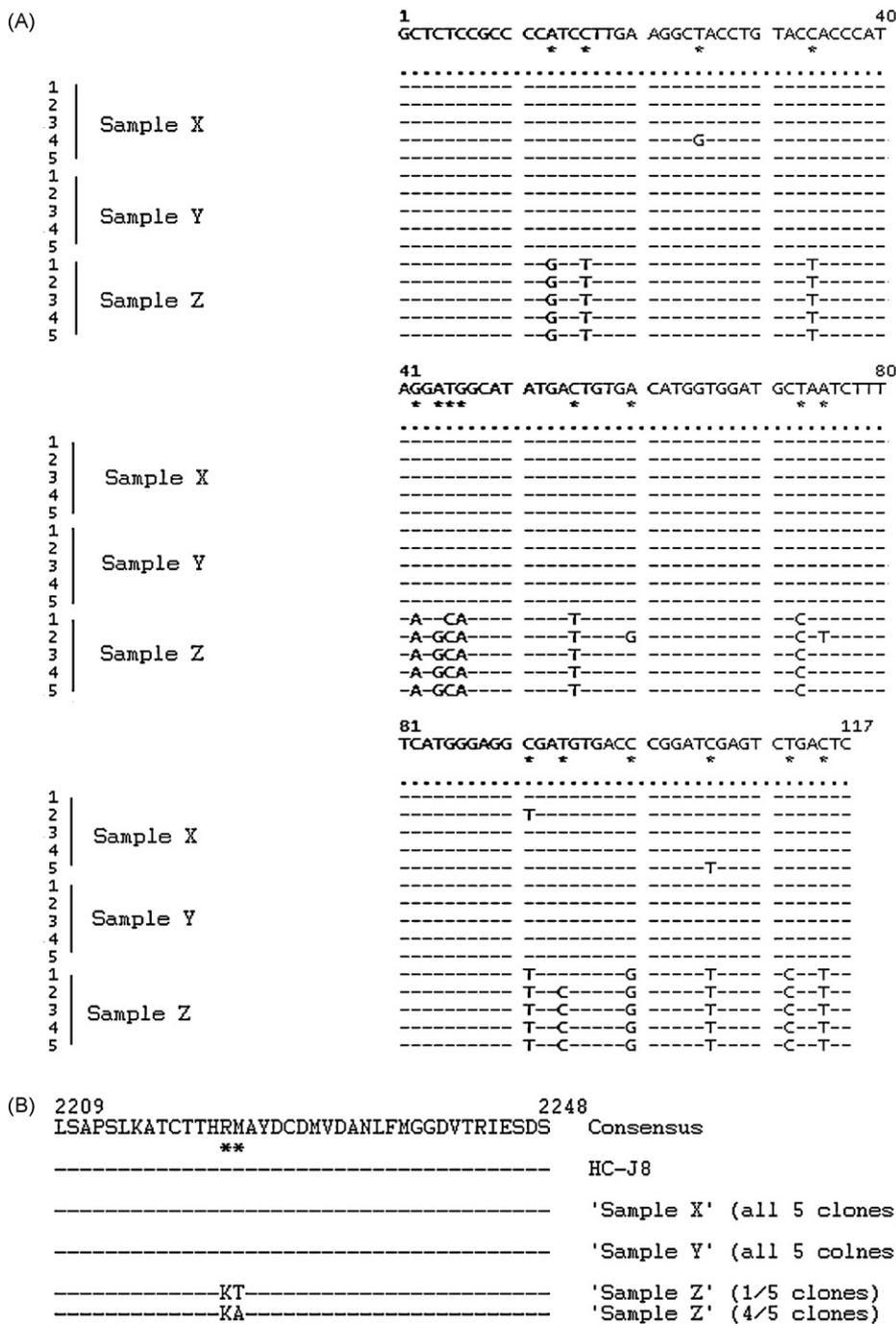


Fig. 1. HCV 2b ISDR quasispecies in Samples X, Y and Z. The consensus sequence is shown at the top; asterisks denote discordance from the consensus. (A) Nucleotide sequence alignment, nucleotide 1 relates to position 6627 of the HCV 2b genome (Genbank EF103925–EF103939). Numbers adjacent to sample names represent individual clones. (B) Consensus amino acid sequence alignment.

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5 weeks after the cessation of antiviral therapy, and genotyping indicated HCV genotype 2b infection. The original pre-treatment specimen and a post-treatment specimen were genotyped, and the 5a-to-2b genotype switch was confirmed. To date, the patient has not been retreated for hepatitis C.

Three serum samples, tested with the patient's informed consent, were available for further studies: Sample X, drawn 5 year prior to anti-HCV treatment; Sample Y, during week 22 of treatment; Sample Z, 5 week after therapy was stopped. Direct nucleotide sequencing of the NS5B-encoding region of the HCV genome (Mellor et al., 1995) confirmed that Sample X carried genotype 5a HCV, while Samples Y and Z carried genotype 2b HCV. Sample Z tested with a commercially available serotyping test (Murex HCV Serotyping 1–6 Assay, Abbot Laboratories) indicated that it carried antibody to genotype 5 HCV. These data are summarized in Table 1.

To determine if genotype 2 HCV was present in sera collected prior to antiviral therapy, a sensitive method to identify minority HCV genotypes (to 0.01% of the total viral population, when the HCV serum load is >1000 IU/ml) was used (Buckton et al., 2006). Briefly, amplicons from the 5'NCR region were treated with *BsmA I*, which selectively digests genotype 5 products. Residual (undigested) amplicons were cloned and subjected to real time nucleotide sequencing using Pyrosequencing. Of 15 clones screened, two could be identified to belong to genotype 2, confirming its pre-existence prior to treatment, while the other clones were genotype 5-derived. To assess the relative proportion of HCV genotype 2 in Sample X, 5'NCR amplicons were cloned and sequenced without prior *BsmA I* digestion. Three of 83 clones were found to be derived from genotype 2, while the others were genotype 5-derived. Thus, genotype 2 HCV comprised approximately 4% of the total population. As the HCV viral load of Sample X was  $1.1 \times 10^6$  IU/ml (tested by the COBAS Taqman HCV assay), genotype 2 could be estimated to be present at a concentration of 44,000 IU/ml.

Previous studies have revealed that the HCV NS5A protein represses the interferon-induced double-stranded RNA-dependent protein kinase (PKR). The PKR-interacting domain of NS5A encompasses what has been termed the interferon sensitivity-determining region (ISDR), which is located between codons 2209–2248 of the NS5A gene. Mutations in the ISDR may account for variability in the host's response to interferon-based therapy (Hofmann et al., 2005). To determine if the genotype 2b HCV that emerged in the patient bore an interferon- $\alpha$ -resistant ISDR genotype, a 323-base pair fragment of the ISDR with flanking sequences was amplified from the three study samples. Oligonucleotide primers specific to the ISDR of genotype 2b HCV were designed to facilitate its amplification under conditions of genotype 5a excess (as in Sample X). Primer sequences were: 5'-AGCCCTGAACCGGACACCGAG-3' (positions 6499–6519 of the HCV genome, based on the HC-J8 HCV strain (Okamoto et al., 1992)) and 5'-GTACTCTGATGGTATGGAAGG-3' (positions 6802–6822). Amplicons generated from these reactions

were cloned as previously described (Buckton et al., 2006), and five from each reaction were sequenced using conventional dideoxy sequencing. Sequence data were compared with published ISDR sequences of genotype 2b HCV strains isolated from reported interferon non-responders (Genbank accession numbers: AY232740–AY232748) (Tanabe et al., 2005). Alignments of the nucleotide and the derived amino acid sequences generated from the clones are presented in Fig. 1, which shows post-treatment emergence of genotype 2b HCV ISDR bearing the R2222K and M2223A/T mutations.

This investigation has revealed that an occult minority genotype HCV can emerge in the course of anti-HCV therapy involving pegylated interferon combined with ribavirin, leading to a poor virological response. In the patient studied, the emerging minority genotype was 2b, which is regarded as being less refractory to antiviral treatment than genotype 5a (Mangia et al., 2005), against which an aggressive and sustained therapeutic regimen was instituted. The type 2b HCV that emerged during therapy not only established dominance, but also acquired mutations in the ISDR which are associated with refractory responses to interferon. To confirm whether the R2222K and M2223A/T mutations are responsible for attenuation of the interferon response, functional studies of the NS5A protein would be required.

It was previously reported that an HIV-1 and HCV co-infected patient who, while receiving aggressive antiviral therapy for chronic genotype 1b infection, became re-infected sexually with HCV of genotype 2b (den Hollander et al., 2005). Whether that strain carried determinants of IFN- $\alpha$  resistance, as in our patient, was not studied. These two cases of intra-therapy HCV genotype-switching to genotype 2b in HIV/HCV co-infected patients raise the question of whether the coinfecting state potentiates such switching.

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