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Bányai, Mária Takács

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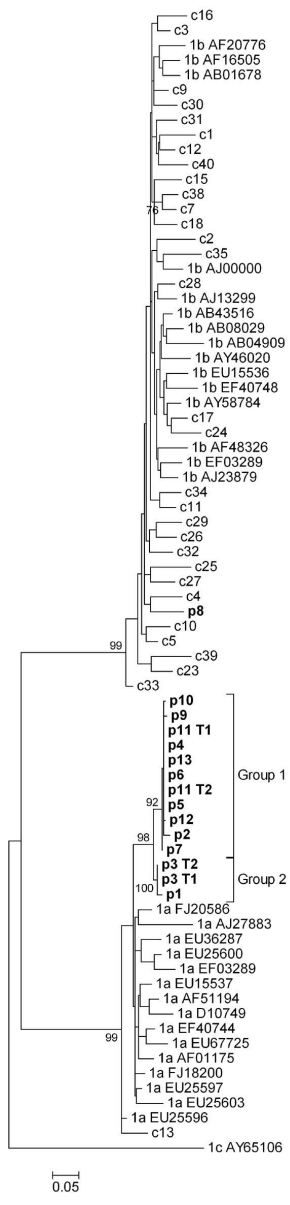


Phylogenetic Investigation Of Nosocomial Transmission Of Hepatitis C Virus In An Oncology Ward

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Keywords:	hepatitis C, NS5B region, envelope region, nosocomial infection, phylogenetic analysis

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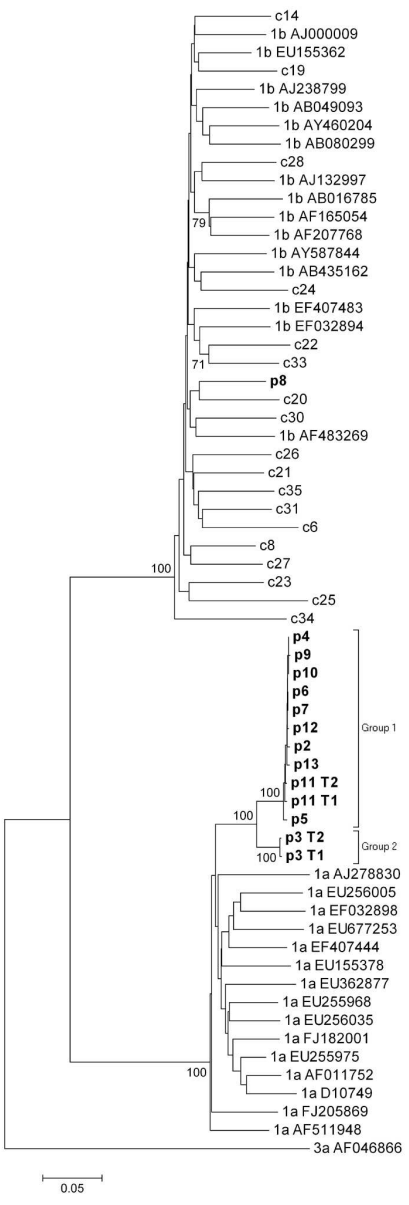


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 7 p11_T1 GTYVSGGSTGRTVAGLTGFFFTAGPQQK
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 14 p2 GTYVSGGSTGRTVAGLTGFFFTAGPQQK
 15 p12 GTYVSGGSTGRTVAGLTGFFFTAGPQQK
 16 p11_T2 GTYVSGGSTGRTVAGLT**E**FFFTAGPQQK
 17 p13 **E**TYVSGGSTGRTVAGLTGFFFTAGPQQK
 18 p3_T2 **S**TYVTGG**S**AAR**D**AS**G**FAG**L**FAVGAK**Q**K
 19 p3_T1 **S**TYVTGG**S**AAR**D**AS**G**IAG**L**FAVGAK**Q**K
 20 1a_AJ278830 **N**TYVTGG**A**AA**K**GAS**T**FAG**L**FAPGS**R**Q**N**
 21 1a_AF511948 **E**TVVTGG**N**VAR**T**TAG**F**AG**L**FSPGA**Q**Q**K**
 22 1a_EU256035 **Q**THVTGG**N**DAR**A**AY**G**IAS**L**FTLGAR**Q**N
 23 1a_FJ205869 **E**THVTGG**S**AAR**A**AS**G**FAS**L**FTVGAK**Q**N
 24 1a_D10749 **E**TIVSGG**Q**AAR**A**MS**G**LV**S**LFTPGAK**Q**N
 25 1a_EF407444 **E**THVTGG**S**AA**H**TT**S**TF**S**RLFTPG**P**SON
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4 1 **Phylogenetic Investigation of Nosocomial Transmission of**
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6 2 **Hepatitis C Virus in an Oncology Ward**
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13 6 **Ágnes Dencs¹, Andrea Hettmann¹, Tibor Martyin², Csilla Jekkel¹, Tivadar Bányai²,**
14 7 **Mária Takács¹**
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Running title: Transmission of HCV in an oncology ward

Abstract

Nosocomial hepatitis C virus (HCV) infections have been reported from different health-care settings worldwide. Twenty patients, treated at the same oncology department, with no previous record of hepatitis C infection, tested positive for anti-HCV antibodies between November 2007 and June 2008. Twelve of the newly infected patients were found to be HCV RNA positive. The common origin of the infections was assumed. To investigate the relatedness of the detected viral strains phylogenetic analyses were performed using sequences from the NS5B and E1/E2 genome regions. A patient carrying HCV for years, was also involved in the study. She was treated at the same oncology department and was considered a possible infectious source. The previous HCV carrier harboured subtype 1b, while all other patients were infected with subtype 1a. Sequences from the 12 newly infected patients formed two groups. The viral sequences within the groups were very closely related. A greater evolutionary distance was observed between the two groups; however, their relatedness could be demonstrated by sequences from both regions with high statistical support. The results indicated that nosocomial transmission occurred. The phylogenetic analyses suggested that the viruses originated from a common source, possibly a patient carrying highly divergent variants. This presumed infectious source could not be identified in the course of this study. The genotype distribution of Hungarian control sequences included in the analysis confirmed this conclusion, since HCV genotype 1a was found to be relatively uncommon.

Keywords: hepatitis C, NS5B, envelope region, nosocomial infection, phylogenetic analysis

Introduction

Hepatitis C virus (HCV) is a member of the *Hepacivirus* genus within the *Flaviviridae* family. HCV infection is a major public health issue worldwide. An estimated 170 million people are infected chronically (WHO, 1997). Chronic infection with HCV can lead to chronic liver disease, cirrhosis and hepatocellular carcinoma. In countries where the screening of donors has been introduced, acute hepatitis C virus infections related to the transfusion of blood or blood products and transplantation of solid organs have become rare. In developed countries, injection drug use accounts for most new HCV infections (Alter, 1999, Esteban et al., 2008). Other potential risk factors include birth to an infected mother, occupational exposure, sex with an infected partner and commercial tattooing (Indolfi and Resti, 2009, Henderson, 2003, Wejstal, 1999, Haley and Fischer, 2003). Health-care associated HCV infections also occur. Outbreaks of hepatitis C have been reported from haemodialysis units, oncology and hematology wards and different inpatient and ambulatory care settings. Most of these involved patient to patient transmission, occasionally affecting a large number of patients (CDC, 2003, Forns et al., 2005, Pekova et al., 2007, Sepkowitz, 2004). Usually, infections are caused by breaches in basic infection control practices by medical staff, such as the reuse of disposable needles and syringes, which can lead to the contamination of infusion equipment or multiple-dose medication vials (Williams et al., 2003). Sepkowitz reviews epidemics in oncology wards and he raises the possibility that HCV can be found in saliva and tears, and might have been transmitted via exposure to these secretions. The possibility that an infected health care worker might serve as the source of infection was examined in most studies and was excluded in all cases. Blood transfusion was also excluded as the source of the epidemics (Sepkowitz, 2004).

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3 1 Phylogenetic analysis of viral sequences obtained following such outbreaks can provide
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5 2 molecular evidence for the relatedness of infections, identify or exclude the possible sources
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7 3 and may help elucidate the events that occurred. Many molecular epidemiological studies on
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9 4 hepatitis C outbreaks have utilized either the fast changing first hypervariable region (HVR1),
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11 5 or partial sequences of the envelope region containing the HVR1 (Izopet et al., 1999, Silini et
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13 6 al., 2002). The more slowly evolving NS5B region has been shown to be best suited for
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15 7 genotyping and subtyping HCV (Laperche et al., 2005), but can also provide enough
16
17 8 information to confirm relatedness of viral strains (Carneiro et al., 2007). However, better
18
19 9 resolution may be achieved by the analysis of the E2 region - including HVR1 – in some
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21 10 cases, where sequences from the recipient are obtained shortly after infection (Bracho et al.,
22
23 11 2005). Some compare or combine the approaches above, or perform quasispecies analysis of
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25 12 envelope region sequences, a method which has significantly improved the understanding of
26
27 13 the transmission of virus variants between donor and recipient (Esteban et al., 1996, Bracho et
28
29 14 al., 2005, Hmaïed et al., 2007). Utilization of longer sequences of both regions has been
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31 15 suggested for improving statistical support of results (Casino et al., 1999). In this paper a case
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33 16 is presented when phylogenetic analysis of sequences outside the first hypervariable region
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35 17 reveal a more distant relationship between transmitted viral sequences.
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43 18 In 2007 and 2008 several patients of the oncology department of a hospital in Hungary
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45 19 returned for treatment or control with elevated ALT levels. Patients treated at the department
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47 20 were screened for anti-HCV antibodies, and twenty patients with no previous history of HCV
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49 21 infection were found to be positive. The possibility of nosocomial transmission was
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51 22 investigated by phylogenetic analyses of partial sequences from both the NS5B and E1/E2
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53 23 regions. The study was approved by an institutional review committee and performed in
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55 24 accordance with Hungarian law. All patients gave their informed consent in writing.
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Materials and methods

Patients and samples

Serum samples of 21 anti-HCV positive patients of the oncology ward were received for the detection of viral RNA, genotyping and phylogenetic analysis. Twenty patients were tested positive for anti-HCV antibodies between November 2007 and June 2008 with no history of previous HCV infection, and one patient had been known to be anti-HCV positive at least since 1998. Serum samples were collected between March and November of 2008, when the patients returned to the hospital for treatment or control. All patients were female, their average age was 57.7 years (range 27-76). Samples from 40 Hungarian HCV carriers from different parts of the country were used as controls (19 male and 21 female, average age 50.2, range: 20-79), including 6 local controls within 150 kilometers from the hospital. All samples were stored frozen at -20°C until the analysis.

Detection of HCV RNA

Nucleic acids were extracted from 200 µl serum using QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Viral RNA was reverse transcribed using ImProm-II Reverse Transcription System (Promega Corp., Madison, WI) with random hexamers and in the presence of 20 units of Rnase inhibitor. 10 µl of the cDNA was used for amplifications in each case.

Table 1 shows the regions and primers used for amplification and sequencing.

Nested primers published by Khan et al. (2004) were used with modifications to detect the conserved 5'UTR regions of all known genotypes of hepatitis C virus. Primers specific for the NS5B region of the HCV genome were designed to detect genotype 1 viruses. PCR specific for the envelope region was performed with primers described by Ray et al. (2000). The

1 primers amplify a 1026 nucleotide long product that contains the carboxy-terminus of the core
2 gene, the complete E1 region and a segment of the E2 region including HVR1.

3 All bands were detected in 2% agarose gel with ethidium bromide staining under UV
4 illumination.

5 Sequencing of PCR products

6 PCR products were purified with Viogene PCR Advanced kit (Viogene, Taipei, Taiwan)
7 using the spin column method. Purified PCR products were sequenced using the DYEnamic
8 ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Little Chalfont
9 Buckinghamshire, UK). Purification of sequencing reactions were carried out according to the
10 ethanol precipitation protocol supplied with the kit. Reaction products were separated and
11 analyzed on a MegaBACE 1000 capillary sequencer (Amersham Biosciences, Little Chalfont
12 Buckinghamshire, UK).

13 Phylogenetic analysis

14 The following hepatitis C virus genotype 1a and 1b sequences were included in the
15 phylogenetic analysis: 1a isolates EU677253, AJ278830, EF032898, EU155378, FJ205869,
16 EU255968, FJ182001, EU256005, EU256035, EU362877, AF511948, AF511949, D10749,
17 EU256005, EF407444, and 1b isolates AY587844, AB435162, EF407483, AF165054,
18 AB049093, EF032894, EU155362, AJ132997, AF483269, AJ000009, AY460204,
19 AF207768, AB080299, AB016785, AJ238799. Sequences were aligned with the MultAlin
20 program (Corpet, 1988). The nucleotide substitution model that described the data best was
21 chosen by ModelTest (Posada and Crandall, 1998) using PAUP* (Swofford, 2002) as
22 implemented in FindModel. Phylogenetic trees were constructed by the Maximum Likelihood
23 method using PhyML 3.0 (Guindon and Gascuel, 2003). The starting tree was computed by

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3 1 PhyML using BIONJ (Gascuel, 1997). Subtype 1c isolate AY651061 was used as an
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6 2 outgroup. Bootstrap analysis with 1000 replicates was performed to confirm tree topology.
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8 3 Evolutionary distance calculations (nucleotide substitution model: Kimura 2-parameter) were
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10 4 carried out using MEGA software version 4.0 (Tamura et al., 2007).
11
12 5 Patient and control sequences obtained in this study were deposited in GenBank under the
13
14 6 following accession numbers: patient sequences from the NS5B region (FN666627-
15
16 7 FN666641), E1/E2 region (FN667677-FN667690), control sequences from the NS5B region
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18 8 (FN666642- FN666672), E1/E2 region (FN667691-FN667708), UTR region (FN668896,
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20 9 FN668897).
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Results

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8 3 The serum samples of 21 anti-HCV positive patients were tested for the presence of HCV
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10 4 RNA using nested PCR with primers specific for the conserved 5'UTR region. Viral RNA
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12 5 could be detected in the sera of 12 of the newly infected oncology patients. The patient with
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14 6 previous HCV infection (patient 8) was also HCV RNA positive, thus was considered a
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16 7 putative source patient in the possible transmission event(s). Antiviral treatment was begun in
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18 8 the case of six HCV RNA positive patients. The other patients could not be treated because of
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20 9 metastasis or other diseases. Details of the 13 HCV RNA positive patients are shown in Table
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27 11 For the purpose of genotyping and phylogenetic analysis, a segment of the NS5B region from
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29 12 the HCV RNA positive patients was amplified and sequenced. Analysis of a 500 nucleotide
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31 13 long segment of the NS5B region was performed using sequences obtained from the 13
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33 14 patients of the oncology, 31 unrelated controls from different regions of Hungary and 30
34
35 15 genotype 1a and 1b isolates from GenBank (Fig. 1). A rooted tree was constructed using the
36
37 16 General Time Reversible model (gamma shape parameter: 0.397, proportion of invariant sites:
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39 17 0.265). The tree showed that all 12 *de novo* infected patients were infected with subtype 1a
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41 18 viruses. Patient 8 was infected with subtype 1b, and was excluded as a possible source patient.
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43 19 The genotype 1a sequences from the outbreak formed two groups with high statistical
44
45 20 support. Group 1 consisted of the closely related viral sequences from 10 newly infected
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47 21 patients (mean evolutionary distance: 0.003) (Table 3a). Five sequences were completely
48
49 22 identical, and only the sequence from patient 2 differed from the others by more than 3
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51 23 nucleotide positions. The sequences from the two other patients were clustered on a separate
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53 24 branch (Group 2). These sequences were separated from Group 1 with a mean evolutionary
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55 25 distance of 0.021. They differed from each other in three nucleotide positions, all three

1 changes also appearing on the amino acid level. Groups 1 and 2 were joined together on the
2 tree by a common branch, which appeared in 98% of bootstrap replicates, suggesting a
3 possible relatedness. The 12 sequences appeared as a cluster that was separated from subtype
4 1a isolates used for constructing the tree by a mean evolutionary distance (0.061) similar to
5 those observed between pairs of independent HCV isolates of the same subtype (mean for
6 subtype 1a: 0.050).

7 To see whether another, faster evolving region also supports this grouping, PCR was
8 performed to amplify a portion from the more variable E1/E2 region of the virus, which
9 included HVR1. Products could be obtained for only 12 patients. Patient 1 could not be
10 included in this part of the study, because the sample did not give a positive result with the
11 E1/E2 primer set even after several attempts. PCR products from 12 other samples were
12 sequenced.

13 The alignment of the 81 nucleotide long HVR1 sequences, often used to demonstrate
14 phylogenetic inference, from the oncology patients infected with 1a subtype strongly
15 supported the existence of Group 1 with only 5 sporadic substitutions in the 10 sequences, 4
16 of them nonsynonymous. No close relationship of the viruses infecting Group 1 patients and
17 patient 3 could be observed. Their HVR1 sequences differed in 12-15 amino acid residues,
18 similarly to unrelated control and GenBank isolates (Fig. 2).

19 However, when the complete determined sequence (positions: 887-1813 nt) was used to
20 construct a phylogenetic tree, it showed a similar picture to the one based on NS5B (Fig. 3).
21 Again, the General Time Reversible model fit the data best (gamma shape parameter: 0.597,
22 proportion of invariant sites: 0.266). The sequence from patient 8 was clustered together with
23 1b subtype HCV isolates. Among 1a isolates from the oncology the same grouping could be
24 observed with high statistical support. Table 3b shows the sequence statistics of the envelope
25 region. Sequences in Group 1 were very close genetically, the average distance within the

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3 1 group was only 0.003. Thirteen variable nucleotide positions were observed, 5 of them within
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5 2 HVR1. These resulted in three variable amino acid residues in the E1 protein (3/192, 1.56%),
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7 3 4 within HVR1 (4/27, 14.81%) and one in the short flanking segment of E2 (1/80, 1.25%).
8
9 4 The mean evolutionary distance between Groups 1 and 2 (patient 3) was 0.049 on the
10
11 5 nucleotide level. In 39 nucleotide positions the occurring bases were characteristic for each
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13 6 group, on the amino acid level 18 differences were unique.
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15 7 The pairwise distances separating unrelated GenBank and Hungarian control sequences of the
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17 8 same subtype were also calculated and - like in case of NS5B sequences - they were found to
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19 9 be typically much greater than between the two patient groups (mean for subtype 1a: 0.115).
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21 10 The NS5B and E1/E2 regions from patients 3 and 11 were sequenced twice, 3 months apart
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23 11 (sequences marked T1 and T2). The NS5B regions were found to be unchanged, in the
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25 12 envelope protein region, however, 2 and 3 substitutions could be observed on the nucleic acid
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27 13 level for patients 3 and 11, respectively, causing one change in the amino acid sequence in
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29 14 each patient.
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31 15 The nucleotide sequences from 40 Hungarian HCV carriers determined in this study served as
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33 16 unrelated controls, but also provided information on the genotype and subtype distribution of
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35 17 Hungarian hepatitis C isolates. 38 of 40 viruses (95%) belonged to genotype 1 based on
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37 18 5'UTR sequences. Subtypes within genotype 1 were determined based on NS5B and/or the
38
39 19 envelope region. Where both regions were sequenced (11 controls), the genotyping results
40
41 20 were in complete concordance. A single subtype 1a virus was found among the 38 genotype 1
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43 21 isolates (1/40, 2.5%), all others were subtype 1b. In the case of two control patients only the
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45 22 5'UTR region could be amplified and these viruses were genotyped based on the nucleotide
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47 23 sequences of that PCR product. One of them was shown to belong to genotype 3, the other to
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49 24 genotype 4. Six of the controls were local controls originating from the South-Eastern part of
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3 1 the country, within a 150 km radius of the hospital (c24, c26, c27, c38-40). They all carried
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5 2 subtype 1b viruses.
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For Peer Review

Discussion

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8 3 After several patients at the oncology department of a hospital in Hungary with elevated ALT
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10 4 levels and no previous record of HCV infection were found to be anti-HCV positive,
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12 5 molecular epidemiological investigation of a possible nosocomial outbreak was performed.
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14 6 The oncology ward had a high patient turnover with several thousand patients per year. They
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16 7 were not routinely screened for hepatitis B or C markers, but blood tests including liver
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18 8 function tests were performed regularly to control side effects of cytostatic treatment. Cases
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20 9 of high liver function were further investigated. A notable increase in the incidence of
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22 10 elevated ALT levels among patients returning for control was noticed in January and February
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24 11 of 2008. Until October of 2008, a total of 45 patients were screened for anti-HCV antibodies,
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26 12 and 21 of them were found to be positive. Twenty of them had no history of hepatitis C
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28 13 infection. One patient had been a known virus carrier for years (patient 8), and was considered
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30 14 a possible common source of infections.
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32 15 Serum samples from 21 anti-HCV positive patients were sent for hepatitis C RNA detection,
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34 16 genotyping and phylogenetic analysis. Thirteen samples were HCV RNA positive, including
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36 17 patient 8, the long time carrier. Genotyping showed that *de novo* infected patients were
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38 18 infected with subtype 1a. However, patient 8 carried a subtype 1b virus, and thus was not the
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40 19 infectious source. Phylogenetic analysis of the NS5B region showed that the viruses of 10
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42 20 newly infected patients were closely related (Group 1) and very likely had a common origin.
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44 21 The viruses infecting the two other patients were similarly closely related to each other
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46 22 (Group 2), but were clearly separated from the other 10 isolates by a distance less than usually
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48 23 observed between unrelated strains. The twelve sequences formed a statistically supported
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50 24 group that appeared monophyletic, which suggested they may be associated. Since NS5B
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52 25 evolves relatively slowly, a large segment of envelope region including HVR1 was also

1 amplified and analyzed. Samples of only 12 of the patients gave positive PCR results with this
2 primer set. This occurred either because of a mutation in the primer binding regions, or the
3 lower sensitivity of this method. The results obtained were similar and provided further
4 evidence of a more distant, but common ancestor. GenBank controls also demonstrated that
5 even unrelated isolates may show relatively low genetic distances, especially in the NS5B
6 region. However, pairs of reference sequences with the smallest distances did not appear as a
7 monophyletic group (e.g. isolates FJ182001 and EU255975). The rapidly changing HVR1
8 supported the existence of Group 1, but the only sequences that could be obtained from Group
9 2 (from patient 3) showed no similarity to Group 1 and differed as much from Group 1
10 sequences as unrelated isolates. Two possible interpretations of these results may be
11 considered. One of these is that the common source of infections was a long time HCV
12 carrier, who carried highly divergent quasispecies. A small inoculum was probably transferred
13 from the source patient to the recipients, which may have only contained a fraction of these
14 variants. Different variants became dominant in the different recipients, and diverged further
15 from their ancestral sequence in the months between the infection and the collection of
16 samples. This may be an example of selective transmission of HCV quasispecies, which has
17 been first described in chimpanzee and *in vitro* experiments, where a total of three
18 chimpanzees were infected with the same HCV containing plasma. Of the quasispecies
19 present in the inoculum, the major variant became dominant in two animals and a minor one
20 in the third animal (Hijikata et al., 1995, Sugitani et al., 1998). Selective transmission has
21 been also documented in humans. After a needle stick accident the minor clone in the donor
22 was found to be the most closely related to the major clone detected in the recipient (Saito et
23 al., 2004). In an outbreak at a haemodialysis unit sequences from two recipients showed
24 greatest similarity to a small subset of variants found in the source patient (Bracho et al.,
25 2005). A HCV carrier cardiac surgeon infected several of his patients during surgery, and the

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3 1 sequences recovered from the different recipients were related to different variants in the
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5 2 surgeon (Esteban et al., 1996). The transmission of a single or a small group of variants was
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8 3 observed also in perinatal infection (Manzin et al., 2000).
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10 4 The common source of the infections at the oncology ward may have been another HCV
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12 5 carrier patient (or a health care worker) not identified by the field investigation, thus not
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14
15 6 included in the analysis. This patient was probably missed by the screening for anti-HCV
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17 7 antibodies either because of normal liver functions, or because of remission from or death
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19 8 caused by the patient's primary disease. Several other patients infected recently at the hospital
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21 9 with HCV could have also been missed by the investigation for the same reasons.
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24 10 Unfortunately, there are not sufficient epidemiological data available to determine the order
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27 11 the infections occurred and trace the patient-to-patient route of transmissions.
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29 12 Another possible explanation of the results is that the sequences in Groups 1 and 2 are not
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31 13 related directly. In this case the reason for the homology detected between them is that they
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34 14 originate from a common population, and have an older common ancestor. To investigate this
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36 15 possibility, the viruses of 40 HCV carrier control patients from Hungary unrelated to the
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39 16 outbreak were also genotyped, including 6 local controls from within a 150 km radius of the
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41 17 hospital. Genotyping of the controls showed that subtype 1b is predominant in Hungary in the
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43 18 clinical material studied (92.5%), which is a similar result to that observed by Gervain
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45 19 (Gervain et al., 2003). Only a single subtype 1a virus was found, originating from a different
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48 20 part of the country and showing no notable sequence similarity to viruses of the oncology
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51 21 patients. These results do not support that two cases of nosocomial transmission may have
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53 22 occurred coincidentally from two unrelated HCV subtype 1a carriers both treated at the
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55 23 hospital.
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57 24 In summary the results suggest that transmission of hepatitis C virus occurred in the oncology
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60 25 ward, where invasive procedures were frequent in the therapy of the patients. The infections

1 probably originated from a common source, although the source patient could not be
2 identified. The precise mode of transmission could not be ascertained either, but
3 contamination of a multiple-dose medication vial or infusion equipment could be suspected.
4 After the outbreak was discovered, strict infection control measures were introduced and
5 regularly monitored. After October of 2008 no more new cases were identified. These
6 findings are similar to those published by Silini et al. (2002), Dumpis et al. (2003), and
7 Maillaird et al. (2009)
8 Silini et al. (2002) observed 13 newly admitted patients with anti-HCV seroconversion in a
9 hematology ward. They excluded the role of HCV infected health care workers, and
10 transfusional origin. Patient-to-patient transmission and the role of multidose vials were
11 assumed as a cause of infection. Dumpis et al. (2003) tested 46 HCV infected patients on a
12 paediatric oncology ward. They also excluded the role of HCV carrier health care workers and
13 the role of the blood transfusion. It was concluded that infections could occur outside the
14 patients' room and may relate to some malpractice by the medical staff. Maillaird et al. (2009)
15 tested a large genotype 3a HCV outbreak at an oncology clinic and found that it was a single-
16 source, patient-to-patient outbreak.
17 Molecular methods have been used widely in investigation of hepatitis C virus outbreaks. In
18 the present study sequence analysis of the NS5B region gave the correct grouping of the
19 isolates under investigation with good statistical support. The observed clusters were
20 confirmed by the phylogenetic tree based on a large portion of the envelope region. It seems
21 that in many cases determining partial sequences (about 500 bases or more) of NS5B is
22 sufficient to show or exclude relatedness of virus strains as also applied in an earlier
23 investigation (Dencs et al., 2009). This method has the added advantage that false negative
24 PCR results due to mutations in the primer binding regions are less likely to occur than in
25 case of the more variable E1/E2. The short HVR1 often used in phylogenetic analyses may

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3 1 also be suitable when only a short time has passed since the infection. However, in this study
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5 2 the more distant relatedness of the virus groups did not emerge from the analysis of this
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8 3 region.
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Table 1. Regions of HCV and primers used for PCR and sequencing

Region	Step	Annealing temperature °C	No. of cycle	Direction	Position*	Sequence
5'UTR	Outer	60	35	Sense	43-68	5'-CCC TGT GAG GAA CTA CTG TCT TCA CG-3'
				Antisense	348-322	5'-TGC TCA TGG TGC ACG GTC TAC GAG ACC-3'
	Nested	59	30	Sense	78-103	5'-TCT AGC CAT GGC GTT AGT ATG AGT GT-3'
				Antisense	313-288	5'-CAC TCG CAA GCA CCC TAT CAG GCA GT-3'
NS5B	Outer	58	40	Sense	8312-8332	5'-GGA GKC AAT YTA CCA ATG YTG TGA C-3
				Antisense	9220-9203	5'-GCC GSD ATT GGA GTG AGT TTR AG-3'
	Nested	57	35	Sense	8622-8636	5'-ACG GAK GCT ATG ACY AGG-3'
				Antisense	9187-9171	5'-ACY GCC CAR TTR AAG AGG TA-3'
E1/E2	Outer	58	35	Sense	834-859	5'-GCA ACA GGG AAC CT TCCT GGT TGC TC-3'
				Antisense	2086-2064	5'-GGG CAG DBC ARR GTG TTG TTG CC-3'
	Nested	59	30	Sense	843-868	5'-AAC CTT CCT GGT TGC TCT TTC TCT AT-3'
				Antisense	1868-1848	5'-GAA GCA ATA YAC YGG RCC ACA-3'

*Nucleotide positions are based on the H77 isolate (accession number: AF009606).

Table 2. Details of the oncology patients included in the molecular epidemiological study

Patient no	Age	Primary disease	First detection of elevated ALT	First detection of aHCV positivity	HCV genotype	Additional information
P1	57	Breast cancer	October 2007	November 2007	1a	
P2	53	Breast cancer	April 2008	May 2008	1a	
P3	70	Ovarian cancer	June 2007	January 2008	1a	
P4	70	Breast cancer	February 2008	August 2008	1a	
P5	58	Breast cancer	December 2007	June 2008	1a	
P6	67	Breast cancer	October 2007	May 2008	1a	
P7	56	Breast cancer	August 2008	June 2008	1a	
P8	76	Hodgkin lymphoma	Before October 1998	Before October 1998	1b	Known to be already a HCV carrier before 1998
P9	65	Ovarian cancer	October 2007	December 2007	1a	Elevated ALT in 2003, but anti-HCV negative. Sudden deterioration of liver functions in 2007
P10	55	Breast cancer	January 2008	January 2008	1a	Developed chronic eosinophilic leukaemia in the 8th week of therapy
P11	51	Colonic adenocarcinoma	November 2007	December 2007	1a	
P12	57	Urinary bladder cancer	January 2008	June 2008	1a	
P13	49	Breast cancer	2005	August 2008	1a	

Table 3a. Sequence statistics based on the determined 500 base long segment from the NS5B region

Compared sequences	Nucleotide level			Amino acid level		
	Number of variable positions	Avg. no. of different positions (SEM)	Mean genetic distance (SEM)	Number of variable positions	Avg. no. of different positions (SEM)	p-distance (SEM)
Within group 1	9	1.636 (0.535)	0.003 (0.001)	4	0.727 (0.354)	0.004 (0.002)
Within group 2	3	3.000 (1.717)	0.006 (0.003)	3	3.000 (1.687)	0.018 (0.010)
Between groups 1 and 2	19	10.455 (2.913)	0.021 (0.006)	7	1.864 (0.870)	0.011 (0.005)
Among controls of the same subtype (1a/1b)	96 / 189	23.792 (2.518) / 31.245 (2.489)	0.050 (0.006) / 0.067 (0.006)	18 / 53	3.392 (0.872) / 5.029 (0.857)	0.021 (0.005) / 0.031 (0.005)
Between subtype 1a controls and groups 1-2	112	28.670 (3.632)	0.061 (0.008)	23	5.643 (1.867)	0.034 (0.011)

Table 3b. Sequence statistics based on the determined 927 base long segment from the envelope region

Compared sequences	Nucleotide level			Amino acid level		
	Number of variable positions	Avg. no. of different positions (SEM)	Mean genetic distance (SEM)	Number of variable positions	Avg. no. of different positions (SEM)	p-distance (SEM)
Within group 1	13	2.836 (0.805)	0.003 (0.001)	8	1.600 (0.569)	0.005 (0.002)
Between group 1 and patient 3	53	43.591 (6.529)	0.049 (0.007)	25	21.000 (3.977)	0.070 (0.014)
Among controls of the same subtype (1a/1b)	326 / 471	97.419 (4.815) / 118.640 (5.465)	0.115 (0.006) / 0.147 (0.008)	92 / 162	34.267 (3.439) / 43.480 (3.764)	0.111 (0.011) / 0.161 (0.014)
Between subtype 1a controls and groups 1-2	349	103.359 (6.426)	0.123 (0.009)	104	41.979 (4.737)	0.137 (0.015)

Legends to the figures

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Fig. 1. Phylogenetic analysis of the NS5B region nucleotide sequences. The maximum likelihood tree includes 30 subtype 1a and 1b isolates from GenBank and 31 unrelated sequences from Hungarian HCV carriers. Sequences from the patients of the oncology are displayed in bold (p1-13), Hungarian control sequences are marked by 'c' and a number. Viral sequences from two patients (p3 and p11) were determined at two different time points (T1 and T2).

Fig. 2. Multiple alignment of HVR1 amino acid sequences from patients of the oncology and of selected HCV 1a isolates obtained from GenBank. Positions differing from the first sequence are shown with a black background.

Fig. 3. Phylogenetic analysis of the E1/E2 region nucleotide sequences. The maximum likelihood tree includes 30 subtype 1a and 1b isolates from GenBank and 18 unrelated sequences from Hungarian HCV carriers. Sequences from the patients of the oncology are displayed in bold (p1-13), Hungarian control sequences are marked by 'c' and a number. Viral sequences from two patients (p3 and p11) were determined at two different time points (T1 and T2).