Evolution of Hepatitis C Virus Quasispecies in Children with Chronic Hepatitis C

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Abstract

Background: Hepatitis C virus (HCV) circulates as a mixture of different but closely related genomes: this quasispecies nature could be essential for virus persistence and could induce resistance to interferon therapy. Since little is known on the behavior of HCV quasispecies in children and adolescents with chronic hepatitis C, we analyzed the virus population in six untreated children during a 5-year follow-up.

Methods: Six children aged 1–8 years, infected early in life with HCV, were included in the study. From each of them, 2 or 3 sequential serum samples obtained over a 5-year follow-up period were examined. The HCV quasispecies heterogeneity and diversity in the E2 hypervariable region-1 (HVR-1) were analyzed among samples by the heteroduplex mobility assay, and the distance between variants was estimated by the heteroduplex mobility ratio (HMR).

Results: The HCV population was initially highly homogeneous in all six children. During follow-up, diversification of HVR-1 leading to a more complex viral population occurred in all cases, and was particularly evident in the three older children (HMR: 0.82–0.54). Changes in the HVR-1 sequence occurred without relation to the profile of ALT and HCV-RNA levels.

Conclusions: HCV quasispecies diversification is a common event during chronic hepatitis C in childhood. Host and environmental pressure could be major determinants. The increasing viral heterogeneity could impair the response to antiviral therapy, thus indicating a rationale for early antiviral treatment in children with chronic hepatitis C.

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Introduction

Hepatitis C virus (HCV) infection is characterized by a high chronicization rate. The mechanisms involved in viral persistence are not completely understood, but the quasispecies nature of HCV is thought to play an important role in maintaining and modulating viral replication [1]. The quasispecies is a mixture of different but closely related viral genomes, resulting from high error rates in RNA replication, which are particularly evident in the hypervariable region 1 (HVR1) of the N-terminus of the E2/NS1 region [2, 3]. A rapid selection among these variants would be the survival strategy used by HCV to face environmental changes, essentially related to host immune reactions. In fact patients with impaired immune response harbor homogeneous genomic populations [4–6]. Vertically infected infants represent an interesting model for virus diversification. They exhibit a relatively homogeneous quasispecies during the first few months of life, with a subsequent increase of nucleotide diversity appearing at 6–13 months, likely reflecting the presence of maternal antibody and the evolution of infantile immune response [7–10]. In adults many attempts have been made to correlate the complexity (total number of variants identified in a single sample) and the diversity (average genetic distance among single variants) of quasispecies with the characteristics and evolution of HCV infection [11–13]. In fact, the issue regarding the role, if any, of HCV diversity in the natural course of chronic infection is still controversial, while more agreement exists on a relationship between complexity of quasispecies and development of resistance to interferon treatment [14, 15]. Little is known on the behavior of the viral population in young children and adolescents with chronic hepatitis C. In this study we have analyzed the pattern of the HVR1 sequence in serial serum samples from six untreated children with hepatitis C followed longitudinally, over a 5-year period.

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Patients and Methods

Patients

Six Caucasian children with chronic hepatitis C, four males and two females aged 1–8 years, were included in the study. The diagnosis of chronic hepatitis was based on the persistence of anti-HCV in serum and of abnormal ALT for longer than 6 months. Children had been selected on the following bases: absence of underlying systemic diseases; persistent HCV-RNA seropositivity; duration of follow-up of at least 5 years and availability of at least two well preserved serum samples spaced over this period. Patients were asymptomatic and had come to observation after a serological anti-HCV screening for previous transfusion (two children), for maternal infection at delivery (three cases), after adoption (one case). Every 6 months, the children were visited in the outpatient clinic and underwent serological investigation. None of the children received antiviral treatment. The study was approved by the Ethical Committee of Padua Hospital.

Methods

In each child, two to three serum samples were obtained at different time points, with first and last sample taken approximately 5 years apart, and stored at –80 °C. Anti-HCV was investigated by third generation ELISA (Ortho Diagnostic Systems, Raritan NJ, USA).

HVR1 PCR amplification and cloning were performed as previously described [16]. Briefly, after RNA extraction, a 196-bp fragment containing the E2-HVR1 was amplified by RT-PCR. The first round used 50 pmol of sense primer 5′GGTGCTCACTGGGGAGTCCT3′ and antisense primer 5′CATTGCAGTTCAGGGCCGTGCTA3′. The second round used sense primer 5′TCCATGGTGGGGAACTGGGC3′ and antisense primer 5′TGCCAACTGCCATTGGTGTT3′. The first round of PCR was performed as follows: 10 ml of the cDNA was added to a 40 µl PCR mixture containing 50 pmol of the external, sense primer, 1.5 mM $MgCl_2$, 23.5 mM Tris-HCl (pH 8.3), 35.5 mM KCl, and 1.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT, USA). A "hot start", nested PCR was then performed: the bottom reaction mixture contained 2.5 mM $MgCl_2$, 0.2 mmol of each dNTP, 10 mM Tris-HCl (pH 8.3), 15 mM KCl, 50 pmol of each internal primer and was separated by a wax layer from the top reaction mixture containing 40 mM Tris-HCl (pH 8.3), 60 mM KCl, 1.5 U of Taq polymerase, and 2% of the first round-product. The PCR was performed using 30 cycles with the following cycling parameters: template denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. A single final extension step was done at 72 °C for 3 min to complete the amplification reaction. Analysis of the quasispecies over time was performed by the heteroduplex mobility assay technique (HMA). As already described elsewhere [17], this technique allows to resolve intra-sample sequence heterogeneity via the mismatches between the probe and the target sequence which, proportionally to the number of nucleotide substitutions, cause delayed mobility of the hybrids in non-denaturing gels. Briefly, to generate a probe, the insert of one clone was re-amplified by PCR, purified by column purification from agarose gel (QiaQuik gel extraction kit; Qiagen, Chatsworth, CA, USA), and the purified DNA was end-radiolabeled with T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD, USA) plus [ɔ-^{32}P]ATP. The probe, which represents one of the quasispecies major variants at the first sampling, was hybridized to the heterogeneous PCR product from serum samples obtained at the different time points, and the different viral sequences were then resolved by non-denaturing polyacrylamide gel electrophoresis. Heterogeneous DNA contains nucleotide differences compared to the probe sequence and, thus, the hybrids (referred as heteroduplex) display retarded mobility on non-denaturing gel relative to the probe hybridized to itself (homoduplex).

The genetic distance between variants can be estimated by calculating the heteroduplex mobility ratio (HMR). The HMR is calculated by measuring the distance in millimeters of the heteroduplex band from the origin of the gel and dividing that by the distance of the homoduplex band from the origin of the gel.

Genotyping was performed analyzing PCR products by a reverse-hybridization assay (InnoLipa HCV II, Innogenetics, Zwijnaarde, Belgium), and virus load was determined by a commercial assay kit (Cobas Amplicor HCV Monitor, version 2.0, Roche Molecular Systems, Branchburg, NJ, USA) according to the manufacturer's instructions.

Results

All six patients were infected with HCV genotype 1b; all remained asymptomatic, with a well-compensated liver disease. ALT were persistently abnormal or fluctuating between normality and thrice the upper normal value. HCV-RNA was positive throughout observation, with intra-host levels fluctuating within one log in all but one patient. All the serum specimens investigated were positive for PCR amplification of the E2-HVR1 sequence using the protocol described.

HMR values in relation to epidemiological and serological features at the time of testing are reported in table 1, while figure 1 shows the results of the HMA assay. A common feature recorded in our patients was a highly homogeneous viral population at the first time point, which was represented by a single major variant, corresponding to a unique band in the gel. The analysis of serum samples at the second and third time points clearly shows that diversification of the HVR-1 quasispecies had occurred over time in all six cases, as indicated by the changes in gel shift pattern and the decrease of HMR values.

In particular, in patients 1 and 2 the quasispecies remained homogeneous at the second time point but the delayed mobility on the gel, as compared to that of the homoduplex control, clearly indicates that the viral population was highly divergent from the original one. Accordingly, in both patients a consistent decrease in HMR values was observed between the first and the second time points (Table 1). On the other hand, in patients 3, 4, 5, and 6, not only the circulating variants were genetically divergent from the original, but also the number of variants increased over time as indicated by the wideness of gel shift pattern and the increased number of bands, respectively. The

Table 1
Epidemiological, biochemical and virological features recorded in the six children at the time when the heteroduplex mobility ratio (HMR) was investigated.

Case No. (sex)	Source of infection	At each HMR assay				HMR
		Age (years)	Duration of infection[a]	ALT N < 50 (IU/l)	Virus load (IU/ml)	
1 (M)	Unknown	6	2	73	3.6×10^5	1
		12	8	38	2.3×10^5	0.70
2 (M)	Transfusion	8	8	111	1.8×10^5	1
		13	13	49	2.2×10^5	0.54
3 (F)	Mother	4	4	44	2.1×10^5	1
		9	9	64	2.5×10^5	0.93
4 (F)	Mother	1	1	142	0.5×10^5	1
		6	6	39	0.9×10^5	0.85
5 (M)	Transfusion	7	7	42	3.5×10^3	1
		12	12	52	2.8×10^5	0.82
6 (M)	Mother	1	1	50	0.4×10^6	1
		2	2	145	2.3×10^5	0.96
		4	4	55	2.9×10^5	0.85

All six were infected with HCV genotype 1b. [a] From birth in the case of HCV-infected mother; from transfusion if exposure was known; from first anti-HCV detection in patients with unknown exposure.

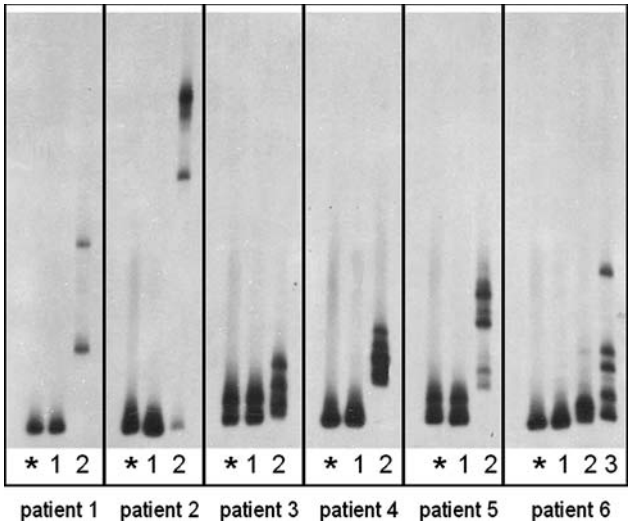

Figure 1. Heteroduplex mobility assay (HMA) of Hepatitis C virus (HCV) quasispecies in the six patients during observation. HVR1 sequences were obtained from at least two consecutive serum samples from each patient. The heterogeneous HVR1 amplification product was hybridized to a radiolabeled probe derived from a clone representing a quasispecies major variant at first sampling, and hybrids were analyzed by non-denaturing gel electrophoresis and autoradiography. For each patient, the first lane contains the probe homoduplex control (*). 1: time point 1; 2: time point 2; 3: time point 3.

greatest genetic distance between the first and second/third time points, expressed in terms of HMR values, was seen in the three oldest children (Table 1, numbers 1, 2 and 5). No relation-ship was detected between ALT values and the patterns of quasispecies diversification over time.

Discussion

The genetic complexity and diversity of the HVR1 are known to evolve during the course of hepatitis C, and to increase with time in the immunocompetent host. Accordingly, the virus quasispecies is more homogenous in agammaglobulinemic subjects, in patients immunosuppressed after liver transplantation, in children with HIV coinfection [18–21], as well as in perinatally infected children during the first months of life [8–10]. Host-selective immunopressure is thought to be the major determinant of intrahost HCV genetic evolution. *Ni* et al. [8] investigated two mother–infant pairs and showed that the evolution of HCV quasispecies was slower in infants than in their mothers, thus supporting the hypothesis that host immune responses play an important role in determining the pace of viral evolution. In this study, we examined the diversification of the HVR-1 quasispecies in six otherwise healthy children with chronic hepatitis C, aged 1–8 years at first testing. In all cases the initial homogeneous pattern evolved to a more complex viral population during a 5-year period. Diversification occurred gradually, as shown in the child tested thrice between 1 and 4 years of age, and reached the highest degree in the older children aged 12–13 years at last testing. Clearly, the small sample size included in this study and the lack of a control group prevent definite conclusions. Nevertheless, our data do not contrast with the concept that intra-host HCV genetic evolution may be a "natural" phenomenon during growth, in parallel with the development of host immunopressure. In addition, it can be speculated that environmental factors could accelerate diversification in adolescents. Evaluating the relationship between HCV quasispecies diversification and source of infection, viremia levels and liver disease was beyond the scope of this study. However, our observations are in keeping with studies in adults, showing that nucleotide sequence diversification occurred without a clear relationship with the ALT profiles and HCV-RNA levels, and with reports in children [10] suggesting that the virus population of HCV-infected newborns remained stable for weeks despite active viral replication. As regards the source of infection we recorded HCV quasispecies diversification both in transfused and in vertically infected children; the higher degree

of diversification in transfused children could rather reflect the older age and the longer duration of infection in those cases.

In adult patients with chronic hepatitis C, the complexity of HCV quasispecies has been regarded as a predictor of response to interferon. Thus, it could be hypothesized that the increasing viral heterogeneity observed in our patients over the years might contribute to reduce the responsiveness to interferon therapy. In fact, in a recent paper *Hartman* et al. [22] found that the response to antiviral treatment was significantly greater among children with a shorter duration of HCV infection, independent of treatment schedule.

In conclusion, the data collected in this small series of children with chronic hepatitis C show that HCV quasispecies diversification occurs throughout childhood and adolescence both in vertically and in horizontally infected patients. The highest degree of diversification was seen in older children transfused perinatally: whether this evolution pattern correlated with the source or the duration of infection or both remains to be evaluated. The increasing complexity of HCV viral population along with increasing age suggests that early treatment of hepatitis C in children should be considered.

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