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Assessment of Genotype and Molecular Evolution of Hepatitis C Virus in Formalin-Fixed Paraffin-Embedded Liver Tissue from Patients With Chronic Hepatitis C Virus Infection

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SUMMARY: Drawbacks of hepatitis C virus (HCV) RNA detection in paraffin-embedded liver tissue have satisfactorily been solved by RT-PCR amplification of the 5′non-coding region (5′NCR). However, detection of this highly conserved region does not provide information on epidemiological or pathogenetic aspects of HCV infection. This study explores whether other functionally important genetic regions of HCV, such as the hypervariable region 1 (HVR-1) and the interferon sensitivity-determining region (ISDR), can be retrieved from paraffin-embedded liver specimens by RT-PCR, and whether the amplified material is suitable for further molecular analyses. RT-PCR amplification of 5′NCR, HVR-1, and ISDR was assessed in RNA extracted from 50 formalin-fixed, paraffin-embedded liver specimens, including 23 needle liver biopsies (11 from patients with non-A, non-B chronic hepatitis diagnosed between 1971 and 1985, 8 from subjects with normal liver histology and 4 from sequential biopsies from a patient with HCV recurrence after liver transplantation), and 27 liver explants from patients undergoing transplantation between 1988 and 1996 (16 with HCV-related cirrhosis and 11 with other disorders). The 5′NCR was successfully amplified in 8 of 11 (73%) non-A, non-B chronic hepatitis biopsies and in all of the specimens from patients with serological documentation of HCV infection. There were no false-positive results. HCV genotype was identified by RFLP analysis of the 5′NCR in the 13 cases analyzed. HVR-1 and ISDR were amplified in 24 of 28 (86%) samples, which were positive for the 5′NCR. Efficient amplification was inversely related to the time of storage. The evolutionary changes of HVR-1 and ISDR were successfully analyzed by direct sequencing of amplificates from the explanted liver and from the sequential liver biopsies in a patient with HCV infection recurrence after transplantation. These observations indicate that paraffin-embedded liver tissue, even when stored for more than 20 years, is appropriate for advanced studies on the molecular biology of HCV. (*Lab Invest 2000, 80:851–856*).

Currently available information on the molecular biology of the hepatitis C virus (HCV) in the liver has been generated mainly by studies carried out in frozen liver tissue specimens (Brambilla et al, 1998; Cabot et al, 1997; De Mitri et al, 1998; Okuda et al, 1999; Sakai et al, 1999). Recently, several authors reported that HCV-RNA sequences may also be recovered and amplified by RT-PCR from formalin-fixed, paraffin-embedded (FFPE) liver tissue (Abe et al, 1998; Akyol et al, 1992; Bresters et al, 1992; Edamato et al, 1996; el-Batanony et al, 1994; Guerrero et al, 1997), even when stored for more than 20 years (Mizuno et al, 1998; Soguero et al, 1999). This approach may be valuable for retrospective studies when frozen liver tissue or serum are not available.

Due to the labile nature of RNA and to the high ribonuclease content of some tissues, degradation of RNA in FFPE specimens often impairs recovery of appropriately preserved target RNA sequences. Several factors, such as delay before fixation, prolonged fixation, and old age of the specimens may favor degradation and prevent further analysis of RNA in FFPE tissues. Different strategies, such as prolonged tissue digestion and amplification of only small RNA targets, have been applied to overcome these shortcomings. However, RNA amplification from FFPE tissues more than 10 years old has rarely been achieved, and only the highly conserved 5′ non-coding region (5′NCR) of HCV has so far successfully been amplified from HCV-infected liver tissue (Abe et al, 1998; Bresters et al, 1992; Edamato et al, 1996; Guerrero et al, 1997; Mizuno et al, 1998; Soguero et al, 1999).

The 5′NCR is a well-conserved region among HCV isolates (Purcell, 1997). Therefore, identification of this region in the liver is a reliable marker of current HCV

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infection, but does not provide relevant information about other aspects that may have epidemiological or pathogenic interest.

In the present study, by following a modified protocol to improve extraction and RT-PCR amplification of RNA from paraffin archival liver tissue, we were able to amplify the hypervariable region 1 (HVR-1) of the E2 gene, as well as the interferon-sensitivity determining region (ISDR) of the NS5A gene, in addition to the 5′NCR. The amplified material enabled further studies, including identification of HCV genotype by RFLP analysis of the 5′NCR, and assessment of the genomic evolution of the virus in the infected liver by direct sequence analysis of other genomic viral regions.

Results

Amplification of 5′NCR and Genotyping by RFLP

Adequacy of RNA extraction from liver specimens was assessed by successful amplification of a 191 nucleotide (nt) fragment of albumin mRNA from the 50 samples tested. The 5′NCR of HCV was amplified from needle biopsies in 8 of 11 (73%) patients with chronic non-A, non-B (C) hepatitis, from liver explants in the 16 patients (100%) who underwent liver transplantation for HCV-related cirrhosis, and from the four sequential needle biopsies obtained from a patient with recurrent HCV infection after liver transplantation. No amplification signal was obtained from liver biopsies from the 3 patients with chronic non-A, non-B hepatitis, in 8 subjects with normal liver histology, or from the liver explants of 11 patients who underwent transplantation for HCV-unrelated liver diseases.

The relationship between the age and type of the specimen analyzed and the result of the RT-PCR amplification of the 5′NCR of HCV in patients with HCV-related disease is shown in Table 1. Amplification was achieved in approximately 50% of the paraffin blocks older than 20 years and in 100% of the blocks of more recent specimens. HCV genotype was investigated by RFLP analysis of the 5′NCR amplified from liver biopsies in 13 patients. HCV genotype 1b was identified in 11 cases, genotype 1a in 1 case, and genotype 1 in the remaining case (HCV subtype could not be determined).

Amplification and Sequence Analysis of HVR-1 and ISDR

After optimization of RT-PCR conditions, amplification of HVR-1 and ISDR was attempted in the specimens where the 5′NCR was successfully amplified. At least 1 μg of total RNA was required for consistent amplification of HVR-1 or ISDR. In preliminary experiments, 190 bp for HVR-1 and 189 bp for ISDR were identified as the optimal target sizes for successful amplification, although longer amplicons were also obtained from liver specimens less than 5 years old (data not shown). The results of amplification of HVR-1 and ISDR are detailed in Table 1, where it is shown that the efficiency of RT-PCR to amplify both regions was quite similar. Successful amplification was related to the time of storage of the liver specimens. The specificity of amplification was confirmed by direct sequence analysis of the PCR products.

To determine whether FFPE liver tissue from infected patients is suitable for evolutionary studies of HCV, one of the patients with HCV-1b–related cirrhosis that developed recurrent infection after liver transplantation was studied. Direct sequence analysis of HVR-1 and ISDR was performed using RNA extracted from the liver explant and from four post-transplant biopsies sequentially obtained during a seven-year follow-up period. The longitudinal intersample variability of each region was estimated by comparing the sequences obtained from sequential biopsies with the consensus sequence from the explanted liver. Data showed that the rate of fixation of mutations in the HVR-1 (0.26 to 7.50×10^{-2} substitutions/nt/year) was greater than in the ISDR (0.08 to 7.05×10^{-2} substitutions/nt/year) (Fig. 1).

Discussion

PCR amplification of nucleic acid sequences from host or microbial agents present in FFPE tissues is a useful tool for retrospective analysis of archival spec-

Table 1. RT-PCR Amplification of the 5′NCR, HVR-1, and ISDR of HCV in Formalin-Fixed, Paraffin Embedded Liver Tissue

Region analyzed	Type of Specimen	Age of the specimen (years)			
		Less than 5	5 to 10	11 to 20	More than 20
5′NCR	Explanted liver	8/8	7/7	1/1	—
	Needle biopsy	2/2[a]	2/2[a]	4/4	4/7
	Total	10/10	9/9	5/5	4/7
HVR–1	Explanted liver	8/8	6/7	0/1	—
	Needle biopsy	2/2[a]	2/2[a]	4/4	2/4
	Total	10/10	8/9	4/5	2/4
ISDR	Explanted liver	8/8	6/7	0/1	—
	Needle biopsy	2/2[a]	2/2[a]	2/4	3/4
	Total	10/10	8/9	2/5	3/4

Results according to the type and age of the specimen.
[a] These biopsies were sequentially obtained from the same patient.

HVR-1

Rate of fixation of mutations (substitutions/nt/year)		AA number	384			412	
Intersample	Acummulated	Liver explant	MVGTWAKVLI	VMLLFAGVDG	TTHVTGAVQG	HTTSGLTSLF	SVGSSQKIQL	VNTNG
7.53×10^{-2}	7.53×10^{-2}	1 month	----------	----------	----------	---R------	----------	-----
5.45×10^{-2}	5.62×10^{-2}	1 year	----------	----------	----------	--IR------	----A----H	-----
0.42×10^{-2}	1.09×10^{-2}	4 years	----------	----------	----------	--IR------	----A-----	-----
1.67×10^{-2}	0.27×10^{-2}	7 years	----------	----------	----------	------A---	N---------	-----

ISDR

		AA number	2209					2248
		Liver explant	PLASSSASQLS	APSLKATCTT	HHDSPDADL	IEANLLWRQE	MGGNITRVES	ENKVVIL
7.05×10^{-2}	7.05×10^{-2}	1 month	----------	----------	---------	----------	----------	-------
$<0.64 \times 10^{-2}$	0.58×10^{-2}	1 year	----------	----------	---------	----------	----------	-------
0.19×10^{-2}	$<0.15 \times 10^{-2}$	4 years	----------	----------	---------	----------	----------	-------
0.19×10^{-2}	0.08×10^{-2}	7 years	----------	----------	---------	----------	----------	-------

Figure 1.

Rates of fixation of mutations and amino acid sequences of the hypervariable region 1 (HVR-1) and the interferon sensitivity-determining region (ISDR) from formalin-fixed, paraffin-embedded (FFPE) explanted liver and sequential liver biopsies from a patient who developed hepatitis C virus (HCV) infection recurrence after liver transplantation for HCV genotype 1b induced cirrhosis. Samples were taken during a 7-year follow-up period. Post-transplant sequences are aligned to the explant sequence. Dashes indicate sequence identity; amino acids that differ from the explant sequence are shown. Intersample and accumulated rates of fixation of mutations were deduced by comparison with the sequences from the previous liver sample or from the explanted liver, respectively.

imens (Mies, 1994). However, RT-PCR amplification of RNA from this material is a complex procedure that may be adversely influenced by several factors, mainly by nucleic acid breakdown during tissue processing and storage (Aurer et al, 1993).

A recent extensive analysis of human and viral genes in FFPE tissue sections showed that amplification can be achieved most consistently by using primers that encompass small (less than 200 bp) RNA targets (Krafft et al, 1997). In agreement with these observations, we showed that a highly sensitive RT-PCR amplification of the 5′NCR of HCV from very old FFPE liver biopsies may be achieved by targeting a short RNA sequence (Soguero et al, 1999). Amplification of either the core or NS3 regions of HCV was not achieved in an early study (Akyol et al, 1992), probably because the size of the targeted RNA was too large (400 and 590 nt, respectively). In the current study, we used a nested PCR procedure in which the outer primers were designed to delimit a PCR product of 237 nt for HVR-1 and 260 nt for ISDR, and the inner primers were designed to delimit products of 190 nt and 189 nt, respectively. The efficiency of the amplification of HVR-1 and ISDR, for similar nt lengths, was equally high for both regions, indicating that selection of a small size target RNA is crucial for successful amplification. Recently, recovery of only small-sized RNA sequences of the Spanish influenza virus from a victim of the 1918 pandemic (Taubenberger et al, 1997) also suggests that the length of the target is more important than the age of the sample for RNA studies in FFPE specimens.

A short time interval between sampling and fixation also seems to be important for efficient amplification (Krafft et al, 1997; Mizuno et al, 1998). The needle liver biopsies analyzed in the present study were immediately fixed in formalin after being taken, and the fixation time was no longer than 24 hours. Successful amplification of HVR1 and ISDR in liver biopsies that were stored for more than 20 years indicates that the RNA was appropriately preserved even in these specimens. The small size of needle biopsies may be another drawback for efficient amplification, which may partially be overcome by intensive extraction of cellular RNA through prolonged proteolytic digestion of the samples (Soguero et al, 1999).

In our study, under optimized RNA extraction and RT-PCR conditions, HVR1 and ISDR amplification was achieved, depending on the age of the material, in 50% to 100% of the samples where 5′NCR amplification was previously demonstrated. Amplification of these regions was slightly less efficient than amplification of 5′NCR, because 100 ng of total RNA was sufficient to amplify the 5′NCR (Soguero et al, 1999) whereas at least 1 μg of total RNA was required for HVR-1 or ISDR. Suboptimal amplification of these regions may be related to its higher genetic variability, which might have caused inadequate matching of primers to target sequence in some samples. However, in contrast to HVR-1 and ISDR, where secondary structure requirements have not been described, the 5′NCR is a highly structured region that includes an internal ribosome entry site element, which is essential for viral transcription (Reynolds et al, 1995; Sáiz et al, 1999). This tight conformational structure of 5′NCR may facilitate preservation of this region in stored specimens.

cDNA amplified from FFPE blocks has been used in complex molecular biology studies, such as Southern blot experiments (Akyol et al, 1992; Guerrero et al, 1997; Krafft et al, 1997) or quantitative analysis (Stanta and Bonin, 1998), and may also be cloned (Taubenberger et al, 1997). As shown in the present study, cDNA amplified from HCV-infected FFPE liver tissue can be used to identify the viral genotype or can be used for evolutionary analysis of HCV, by direct se-

quencing of appropriate HCV genomic regions in sequential liver samples.

Genotype 1b was the most frequently identified HCV genotype by RFLP analysis of 5′NCR amplified from FFPE liver tissue, in close agreement with the results of HCV genotype studies carried out in serum samples from our geographical area (López-Labrador et al, 1997). Identification of HCV subtype was not achieved in 1 of the 13 cases analyzed, therefore, further studies are necessary to evaluate the performance of other genotyping techniques in HCV material retrieved from paraffin-embedded archival specimens.

Demonstration that HVR-1 and ISDR may be amplified and sequenced in HCV-infected FFPE liver tissue deserves further comment, because the biological importance of these regions is increasingly being recognized. The HVR-1 of HCV is a major immunogenic domain, bears major neutralizing epitopes (Kato et al, 1993; Shimizu et al, 1994), and evolutionary changes of this region are believed to have important implications in viral persistence and pathogenicity (Gretch et al, 1996; Kato et al, 1994; McAllister et al, 1998; Tsai et al, 1998). The role of the ISDR in the response to interferon is still a matter of controversy (Brechot, 1999; Enomoto et al, 1996; Hoofnagle, 1997; Sáiz et al, 1998), but this region seems to modulate the interferon transduction pathway by interacting with a cellular protein kinase (Gale et al, 1997, 1998).

Analysis of the genomic evolution of HVR-1 and ISDR in four biopsies taken during a 7-year follow-up period from a patient with post-transplant HCV recurrence showed that the rate of fixation of mutations in the HVR1 was higher than in the ISDR. Although no amino acid replacement was observed in the ISDR, amino acid fluctuations occurred in the HVR-1. These observations indicate that FFPE liver tissue is suitable for evolutionary analysis of HCV in infected patients.

HCV infection recurrence after liver transplantation for HCV-induced cirrhosis is among the most important and unsolved problems of clinical Hepatology. Genetic diversification of HCV, which is usually measured in the HVR-1, seems to play an important role in the pathogenesis of this condition (Gretch et al, 1996). Recent observations suggest that an increased HCV genetic diversification may be associated with a more benign course of recurrent HCV hepatitis (Sánchez-Fueyo et al, unpublished observations; Sullivan et al, 1998). So far, the diversification of HVR-1 in patients with post-transplant HCV recurrence has been studied by analysis of sequential serum samples, but studies in liver tissue, where HCV infection takes place, have not been performed. It has also been shown that HCV populations may be different in serum and liver (Cabot et al, 1997; Sakai et al, 1999). Therefore, studies based on amplification and sequence analysis of HVR-1 in FFPE liver tissue may provide new insight into the role of viral diversification in patients with post-transplant HCV infection recurrence and in other clinical situations.

Materials and Methods

Archival Specimens

Fifty FFPE liver specimens, including 27 liver explants and 23 needle biopsies were studied. Liver explants were obtained from patients who underwent liver transplantation between 1988 and 1996 (16 had HCV-related cirrhosis, 5 had HBV-related cirrhosis, 4 had alcoholic cirrhosis, 1 had primary biliary cirrhosis, and 1 had primary sclerosing cholangitis). HCV-RNA was demonstrated in all of the patients with HCV-induced cirrhosis when their sera were tested retrospectively. Needles biopsies were obtained between 1971 and 1985 from 11 patients with chronic non-A, non-B hepatitis (who subsequently were diagnosed as chronic hepatitis C, with positive anti-HCV and HCV-RNA in serum), and from 8 patients with normal liver histology and no serological evidence of current HBV or HCV infection. Four additional trucut liver biopsies were serially obtained over a period of 7 years from a single patient who developed recurrent HCV infection after liver transplantation for HCV-related cirrhosis.

Isolation of RNA from Tissue

Total cellular RNA was extracted from 10 μm-thick sections by proteolytic enzymatic digestion, as previously described (Soguero et al, 1999). Briefly, tissue was dewaxed with changes of xylene and sections were resuspended in digestion buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% SDS, and 0.2 mg/ml of proteinase K. The incubation was carried out in a water bath at 42° C for 4 days. Nucleic acids were extracted with phenol/chloroform, followed by overnight precipitation in isopropanol and glucogen. The pellet was washed once in 70% ethanol, air-dried, and resuspended in sterile diethylpyrocarbonate-treated water. The total amount of isolated RNA was measured by spectrophotometry at 260 nm.

Amplification of HCV Genomic Regions

HCV-RNA from liver and serum was amplified by RT-PCR using primers derived from the 5′-NCR as previously described (Sánchez-Tapias et al, 1996; Soguero at al, 1999). The HVR-1 was amplified by nested PCR using the following primers: HV7 (outer sense, nt 1396 to 1417) 5′-ACT GGG GAG TCC TGG CGG GCC T-3′; HV5C (outer antisense, nt 1611 to 1633) 5′-TCA TTG CAA TTC AGG GCA GTC CT-3′; HV3B (inner sense, nt 1431 to 1453) 5′-ATG GTG GGT ACC TGG GCT AAG GT-3′; and HV6B (inner antisense, nt 1598 to 1621) 5′-AGG GAA TTC CTG TTG ATG TGC CA-3′. The expected sizes of the PCR products were 237 bp and 190 bp, respectively. The ISDR was amplified using primer SEQ2 (outer sense, nt 6906 to 6925) 5′-AGG CTG GCT AGG GGG TCT CC-3′, IS3 (outer antisense, nt 7145 to 7165) 5′-AGG ATC TCC GCC GCA ACG GAT-3′, IS1 (inner sense, nt 6931 to 6950) 5′-CCT TGG CCA GCT CTT CAG CT-3′ and IS2 (inner antisense, nt 7101 to 7120) 5′-CGA AGC GGG TCA AAA GAG TC-3′. The expected sizes

of the PCR products were 259 bp and 189 bp, respectively. Nt positions are according to Okamoto et al (Okamoto et al, 1992).

Optimized RT-PCR conditions for both HVR-1 and ISDR were similar to those previously described for the 5′NCR (Soguero et al, 1999). Briefly, for cDNA synthesis, 1 μg of total RNA was denatured at 70° C for 5 minutes and cooled on ice. The reverse transcription reaction was performed with 300 U of Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL, Gaithersburgh, Maryland), random hexanucleotides (Boehringer Mannheim, Manheim, Germany), and 20 U of ribonuclease inhibitor (RNAsin; Promega, Madison, Wisconsin) in a final volume of 25 μL. The mixture was incubated for 90 minutes at 37° C, followed by 10 minutes at 95° C. The PCR reaction was performed in a separated tube with 1.5 U of *Taq* polymerase (GIBCO-BRL) in a final volume of 50 μl. PCR parameters were as follows: an initial 5 minutes at 94° C followed by 35 cycles of 1 minute at 94° C, 1.5 minutes at 55° C, and 1.5 minutes at 72° C, with a final 10-minute completion step at 72° C. A 5 μl aliquot was re-amplified in a 50 μl reaction mixture with the following cycling parameters: an initial 5 minutes at 94° C followed by 35 cycles of 1 minute at 94° C, 1 minute at 55° C, and 1 minute at 72° C, with a final 10-minute completion step at 72° C. Amplified products were subjected to electrophoresis on 1.5% agarose gel stained with ethidium bromide.

Amplification of a 191 nt albumin mRNA fragment was performed in every experiment as a housekeeping gene control (Soguero et al, 1999). To prevent contamination, universally accepted guidelines (Kwok and Higuchi, 1989) were strictly followed, including the use of different blades for sectioning each sample and the use of separated rooms for nucleic acid extraction, amplification, and analysis. All RNA extractions and amplifications were repeated on three separate occasions to ensure reproducibility.

HCV Genotype and Sequence Analysis

HCV genotype was determined by RFLP analysis of 5′NCR amplificates, as previously described (Thiers et al, 1997). For sequence analysis, specific HVR-1 and ISDR bands were excised from the gel and purified using the CONCERT Rapid Gel extraction kit (GIBCO BRL, Karlsruhe, Germany), then directly sequenced using the Rhodamine Terminator Cycle sequencing kit (Perkin Elmer Applied Biosystems, Warrington, United Kingdom) in a 310 DNA sequencer (Applied Biosystems, Westerstad, Germany). Sequence editing was performed using Sequence Navigator (Applied Biosystems, Warrington, United Kingdom).

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