American Journal of Pathology, Vol. 141, No. 6, December 1992

Short Communication

Detection of Replicative Hepatitis C Virus Sequences in Hepatocellular Carcinoma

Michael A. Gerber,* Y. S. Carol Shieh,* Kyu-Sik Shim,* Swan N. Thung,† A. Jake Demetris, ‖Myron Schwartz,‡ Gulen Akyol,* and Srikanta Dash,*

From the Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, Louisiana, The Lillian and Henry M. Stratton—Hans Popper Department of Pathology† and the Department of Surgery,‡ Mount Sinai School of Medicine, New York, New York, and the Department of Pathology,‖ Presbyterian-University Hospital, Pittsburgh, Pennsylvania*

Several serologic studies suggest that infection by hepatitis C virus (HCV) may be associated with the development of hepatocellular carcinoma (HCC). Therefore, we examined tumor tissue and/or the surrounding liver of 20 patients for viral sequences by the polymerase chain reaction (PCR). In 12 cases, liver and tumor tissues were separable for extraction. RNA was extracted from frozen tissues and used as a template for reverse transcription followed by double PCR with nested primers for the 5′-untranslated (NT) and nonstructural NS3 regions of HCV. In addition, the tissue extracts were tested by single PCR for X gene and S gene sequences of hepatitis B virus (HBV). NT region sequences of HCV were detected in the available tumor tissue of all anti-HCV-positive patients except for one. Negative (replicative) strands of HCV RNA were found in the same tissues as positive (genomic) strands at almost the same relative amounts, suggesting replication of HCV in the tumor tissue rather than contamination by HCV-positive blood. HBV X and S sequences were demonstrated in two tumors, but were absent from three tumors that were surrounded by liver tissues with HBV X sequences. One patient had nucleic acids of both viruses in tumor tissue. These observations suggest that in addition to HBV, HCV may play a role in the development of hepatocellular carcinoma. (Am J Pathol 1992; 141:1271–1277)

Hepatocellular carcinoma (HCC) is the most common visceral cancer in the world.[1] Multiple lines of evidence indicate that hepatitis B virus (HBV) is causally related to the development of HCC. In the United States, only a fraction (<25%) of HCC appears to be associated with HBV infection.[1,2] Recent studies suggest that chronic infection with hepatitis C virus (HCV) may represent another risk factor for the development of HCC. This hypothesis is based on reports that demonstrate that patients with HCC have a high prevalence of anti-HCV antibodies.[3–13] Unfortunately, however, the available anti-HCV assays have significant numbers of false-positive and false-negative results.[14–18] The serum titer of HCV rarely reaches 10^6 chimpanzee infectious doses in patients[17] and therefore, the virus cannot be detected by conventional techniques. For this reason, we and others developed a modified polymerase chain reaction (PCR) method to detect HCV sequences in serum or liver tissue.[19–23] HCV represents a positive strand RNA virus and probably replicates via negative strand intermediates.[24] Positive (genomic) and negative (replicative) strands of HCV may be distinguished by use of antisense or sense primers, respectively, for reverse transcription to cDNA, followed by double PCR with nested primers, gel electrophoresis of the amplification products, and Southern blotting.[23,24] This method is both sensitive and highly specific and enables us to test selected areas in minute tissue samples for HCV sequences.[22] In this study, we determined (1) whether HCV is present in HCC and the surrounding non-tumorous liver, (2) whether replicative intermediates (negative strand RNA) are detectable in the tissues, and (3) whether the relative

Supported in part by NIH grants DK 40587, CA 54576, and CAGNO grant 535700.

Presented in part at the V International Symposium on Viral Hepatitis in Madrid, January 1992, and a summary will be published in the Journal of Hepatology.

Accepted for publication August 19, 1992.

Address reprint requests to Dr. Michael A. Gerber, Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2699.

quantities of positive and negative strands in HCC and non-tumorous liver are similar.

Material and Methods

Twenty patients (6 females and 14 males, ranging in age from 2–74 years) with HCC were studied. The diagnosis was based on clinical, biochemical, serologic, and histologic criteria (Table 1). Serum anti-HCV testing was done by enzyme immunoassay (Ortho Diagnostics, Westwood, MA) and was considered positive if the optical density was repeatedly higher than 2.5. Serum HBsAg, anti-HBs, and anti-HBc were tested by radioimmunoassay (Abbott Laboratories, Chicago, IL). Liver tissues were obtained from partial hepatectomy specimens (9 cases) or from livers removed before liver transplantation (11 cases) and were tested without knowledge of the etiology of liver disease. Two pathologists (MAG and GA) carefully selected tissue samples by histologic examination of frozen sections to represent liver or HCC, but if possible not both in the same piece. Clear separation of liver and tumor tissues was possible in 12 cases. In four cases, only tumor tissue was available, in two cases only liver tissue, and in two cases liver and tumor tissues were intermingled to such an extent that they could not be separated. RNA was extracted from about 1 mg of frozen tissue and used as a template for reverse transcription (RT) followed by double PCR with nested primers for the 5′ non-translated (NT) and non-structural 3 (NS3) regions of HCV. In addition, DNA was extracted from all samples and tested by single PCR for X and S gene sequences of HBV. Amplification products were demonstrated by Southern blot hybridization with radiolabeled oligomer probes that recognized sequences between the primers.

Extraction of RNA from Liver and Tumor Tissues[22]

Fresh frozen tissues were mixed with guanidine solution (4 mol/l guanidinium thiocyanate, 25 mmol/l sodium citrate, pH 7, 0.5% sarcosyl, 0.1 mol/l 2-mercaptoethanol) and homogenized with a glass homogenizer at 4°C. Sodium acetate and phenol/chloroform were added to the homogenate, and the aqueous phase was collected after centrifugation at 10,000 rpm for 10 minutes. Isopropanol was added, and RNA was precipitated at −70°C for 1 hour. RNA pellets were resuspended in 10–20 μl water and stored in −70°C until use.

Extraction of DNA from Liver and Tumor Tissues

Fresh frozen tissues were mixed with lysis buffer (50 mmol/l Tris, pH 7.5, 10 mmol/l EDTA, 1% SDS) and homogenized with a glass-teflon homogenizer. The mixture was incubated at 55°C overnight after adding proteinase K at 500 μg/ml. Two phenol/chloroform extractions were used to purify DNA followed by one extraction of chloro-

Table 1. *Summary of Patients and Results of Polymerase Chain Reaction for HCV RNA and HBV DNA Sequences*

Case no.	Age (yr)	Sex	Diagnosis	Serum Anti-HCV	Serum HBsAg	Results of PCR on Tissue Extracts					
						HCV NS3		HCV NT		HBV X*	
						Liver	Tumor	Liver	Tumor	Liver	Tumor
1	59	M	Cirrhosis & HCC	+	−	−	−	+	+	−	−
2	64	M	Cirrhosis & HCC	+	−	− (mixed)		+ (mixed)		− (mixed)	
3	62	M	Cirrhosis & HCC	+	−	+	−	+	+	−	−
4	53	M	HCC	+	+	na	+	na	+	na	+
5	58	F	Cirrhosis & HCC	+	−	+	na	+	na	−	na
6	56	M	Chronic hepatitis & HCC	+	−	+	na	+	na	na	na
7	76	M	Cirrhosis & HCC	+	−	+ (mixed)		+ (mixed)		− (mixed)	
8	67	M	Cirrhosis & HCC	+	−	−	−	−	−	na	na
9	54	M	Cirrhosis & HCC	−	−	na	+	na	+	na	−
10	52	M	Cirrhosis & HCC	−	−	+	+	+	+	−	−
11	42	F	Cirrhosis & HCC	−	−	−	−	+	−	−	−
12	74	M	Cirrhosis & HCC	−	−	−	−	+	+	−	−
13	54	M	Cirrhosis & HCC	−	+	na	−	na	−	na	−
14	43	M	Chronic hepatitis & HCC	−	+	−	−	−	−	+	−
15	45	F	Chronic hepatitis & HCC	−	+	−	−	−	−	+	+
16	2	F	Normal liver & HCC	−	−	−	−	−	−	+	−
17	59	M	Cirrhosis & HCC	−	−	−	−	−	−	+	−
18	52	M	Cirrhosis & HCC	−	−	−	−	−	−	−	−
19	65	F	Steatosis & HCC	−	−	−	−	−	−	−	−
20	48	F	Cirrhosis & HCC	−	−	na	−	na	−	na	−

* The PCR results for HBV S gene sequences were identical except for case 14 which was negative for S gene sequences in both liver and tumor. na = not available. mixed = liver and tumor tissues could not be separated.

form. DNA was precipitated at room temperature for 30 minutes by adding two volumes of isopropanol. DNA pellets were resuspended into TE buffer, pH 8.0 (10 mmol/l Tris, 0.1 mmol/l EDTA) and stored in −20°C until use. The quantity of nucleic acid was determined spectrophotometrically at 260 nm.

Preparation of Primers and Probes for PCR

Oligonucleotide primers specific for HCV sequences were synthesized on a Milligen/Biosearch Cyclone DNA synthesizer (Novato, CA) by phosphoramidite chemistry. Primers were deprotected by treatment with ammonium hydroxide for 24 hours at 25°C and examined by electrophoresis on native 20% polyacrylamide gels. Oligonucleotide primers were vacuum dried, resuspended in 200 to 500 μl of water and quantitated by absorbance at 260 nm. Primer sequences were selected from published reports.[22] Primers for the second PCR were selected from within the genomic region amplified by the first PCR. Nucleotide sequences of the oligomers and the amplified product sizes are shown in Table 2.

Preparation and Amplification of HCV cDNA

For cDNA synthesis from positive strand HCV RNA, the outer antisense primers for NS3 or NT sequences (500 ng) were mixed with liver or tumor-derived total RNA (500 ng), denatured at 65°C for 15 minutes and annealed at room temperature (total volume 20 μl). AMV reverse transcriptase (7.5 units) (Promega, Madison, WI) was added and incubated at 42°C for 60 minutes in the presence of 50 mmol/l Tris, pH 8.3, 50 mmol/l KC1, 10 mmol/l $MgCl_2$, 1 mmol/l DTT, 1 mmol/l EDTA, 500 nmol/l dNTP, 250 nmol/l spermidine, and 40 υ RNAsin (Promega). After addition of the second outer primer, (250 ng) PCR was done with Taq DNA polymerase (Promega) under standard conditions (0.2 mmol/l dNTP, 50 mmol/l KCl, 10 mmol/l Tris pH 8.3., 1.5 mmol/l $MgCl_2$, 0.01% gelatin), and carried out for 35 cycles using the following program: denaturation for 1 minute at 93°C, annealing at 50°C for 30 seconds and extension for 1 minute at 72°C. A second PCR with nested inner primers (250 ng each) was done by using 1/10 of the first PCR product as DNA template. The second PCR was carried out as described earlier, except that the extention time was reduced to 30 seconds.

The cDNA synthesis of negative strand HCV RNA was done by using the outer sense primer for NT sequences under the same conditions as described earlier. At the end of reverse transcription, AMV transcriptase was inactivated by boiling the reaction mixture for 60 minutes.[23,24] AMV transcriptase should be eliminated to prevent reverse transcription of positive strand HCV RNA when adding the outer antisense primer for amplification. Therefore, in several experiments cDNA of negative strand HCV RNA was purified by phenol/chloroform and then amplified by double PCR using the NT primer pairs as described earlier. This method also eliminated any residual AMV transcriptase and prevented reverse transcription of positive strands during amplification. Finally, omission of the second primer after reverse transcription resulted in no amplification indicating that the amplification was strand-specific.

The relative quantities of both strands of HCV RNA NT sequences were compared in three representative cases in which both HCC and non-tumorous liver tissues were available. For this purpose, serial ten-fold dilutions of 500 ng RNA extracted from the tissues were tested by reverse double PCR. The serum of two of these three patients was available, and extracted RNA (500 ng) was serially diluted for reverse double PCR.

Table 2. *Nucleotide Sequences of the Oligomers and the Amplified Product Sizes*

Region	Primer	Oligomer sequence	Product size
HCV NS3	1st PCR: sense	5′-GCAATACGTGTGTCAC-3′	589 bp
	antisense	5′-GACATGCATGTCATGATGTA-3′	
	2nd PCR: sense	5′-GCATCTACAGATTTGTG-3′	338 bp
	antisense	5′-CTTCCACATCTGGTCC-3′	
	Probe	5′-CTTTACAGGCCTCACTCATATAGATGCCCACTTTCTATCCCA-3′	
HCV NT	1st PCR: sense	5′-CTGTGAGGAACTACTGTC-3′	219 bp
	antisense	5′-CACTACTCGGCTAGCAGT-3′	
	2nd PCR: sense	5′-CACGCAGAAAGCGTCTAG-3′	142 bp
	antisense	5′-TTGATCCAAGAAAGGACCC-3′	
	Probe	5′-AGTATGAGTGTCGTGCAGCCTCCAGGA-3′	
HBV X	1st PCR: sense	5′-CTGGATCCTGCGCGGGACGTCCTT-3′	228 bp
	antisense	5′-GTTCACGGTGGTCTCCAT-3′	
HBV S	1st PCR: sense	5′-GTTCTTCTGGACTACCAAGG-3′	330 bp
	antisense	5′-CAGACTTGGCCCCCAATACC-3′	
	Probe	Full length HBV genome cloned into pBR 322	

Amplification of HBV X and HBV S DNA

Amplification of HBV X DNA and of HBV S DNA by single PCR was done as described by us.[25]

Analysis of Amplified Samples

DNA segments amplified by PCR were visualized in 2% agarose gels and transferred to a Zeta probe nylon filter. The filters were hybridized with HCV synthetic oligoprobes that recognized sequences between the two inner primers (listed earlier). The probes were end-labeled with ^{32}P at the 3'-end by terminal deoxynucleotidyl transferase. The hybridization conditions for HCV probes were the same as previously described[22]; except that the filters were washed at 37°C with 4X SSC/0.1% SDS. The full-length HBV DNA probe was labeled with ^{32}P deoxycytidine triphosphate by nick translation (Nick Translation Kit, Promega, Madison, WI). The filters of HBV DNA were incubated under the same condition at 42°C, but washed at 55°C with 2X SSC/0.1% SDS. Autoradiography was carried out for a few hours to overnight at −70°C.

Extensive precautions were taken to prevent contamination including physical separation of nucleic acid preparation from amplification and use of positive pressure pipettes as described previously.[25] Negative (normal liver extracts, no template, or no AMV transcriptase) and positive controls were included in all experiments. All PCR runs were repeated to ensure reproducibility.

Results and Discussion

In this study, we completed reverse transcription/double PCR of HCV NT and NS3 sequences on liver and/or tumor extracts of 20 cases. We also completed single PCR for HBV X and S sequences on these tissues. The sensitivity of single PCR alone was adequate for the demonstration of HBV.[25] However, for optimal sensitivity of HCV detection, double PCR was needed as shown previously in our laboratory.[22] Southern blot hybridization with oligomer probes assured the specificity of the amplified products which were visible on agarose gels. As additional specificity controls, all specimens were tested twice and positive and negative controls were included in all PCR runs.

Among the 20 patients examined, liver and/or tumor tissues of 11 cases contained amplifiable NT sequences of HCV, whereas NS3 sequences were detected in only 7 cases (Table 1, Figure 1). This finding confirms previous reports that the NT region represents a highly conserved area of the viral genome.[26–30] Amplification of this region gives more consistent results than other regions of

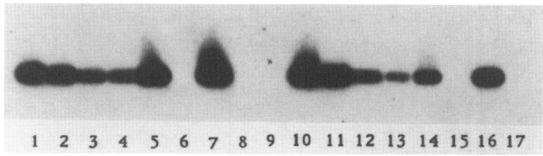

Figure 1. *PCR amplification of HCV NT positive and negative strand RNA in liver and tumor tissues. Viral positive strand (genomic) and negative strand (replicative) RNAs were examined by double PCR using NT antisense and sense primers, respectively, for reverse transcription. After double PCR, the products were hybridized with end-labeled oligomer. Lane 1: Positive strand HCV RNA of liver, Case No. 10. Lane 2: Positive strand HCV RNA of tumor, Case No. 10. Lane 3: Positive strand HCV RNA of liver, Case No. 1. Lane 4: Positive strand HCV RNA of tumor, Case No. 1. Lane 5: Positive strand HCV RNA of liver, Case No. 11. Lane 6: Positive strand HCV RNA of tumor, Case No. 11. Lane 7: Positive strand HCV RNA of tumor, Case No. 4. Lane 8: Negative control for positive strand HCV RNA. Lane 9: Lambda DNA marker. Lane 10: Negative strand HCV RNA of liver, Case No. 10. Lane 11: Negative strand HCV RNA of tumor, Case No. 10. Lane 12: Negative strand HCV RNA of liver, Case No. 1. Lane 13: Negative strand HCV RNA of tumor, Case No. 1. Lane 14: Negative strand HCV RNA of liver, Case No. 11. Lane 15: Negative strand HCV RNA of tumor, Case No. 11. Lane 16: Negative strand HCV RNA of tumor, Case No. 4. Lane 17: Negative control for negative strand HCV RNA.*

the HCV genome that show extensive sequence diversity between different HCV isolates.[20] In case 11, the liver tissue, but not the tumor tissue, contained amplifiable NT sequences. As demonstrated later by serial dilution studies, some liver tissues contain larger amounts of HCV RNA than tumor tissues although we cannot exclude the possibility that deletions or mutations of the HCV genome occurred in the malignant tissues. These questions will be addressed by cloning and sequencing the entire HCV genome from both liver and tumor tissues.

All cases with positive strand (genomic) HCV RNA, were also tested for negative strand (replicative) RNA of the HCV NT region in both liver and tumor tissues. Positive strand RNA and negative strand RNA were always found together in both liver and tumor tissues (Table 3, Figure 1). Serial dilution of RNA extracts of tissues from three cases showed that the titers of both strands of the HCV NT region were the same in liver and tumor tissues in two cases and ten times less in the tumor in the third case (Table 4). The titer of the negative strand was ten

Table 3. *HCV NT Positive and Negative Strand RNA in Liver and Tumor Tissues as Determined by PCR*

	Number of Tissues With HCV NT RNA	
Tissues	Positive strand	Negative strand
Liver	7	6†
Tumor	6*	6
Mixed liver and tumor	2	2
Total	15	14

* Tissue of one HCC was negative for HCV NT positive and negative strand RNA while the surrounding liver was positive for both strands.

† In one case, there was not enough liver tissue available to test for negative strand RNA.

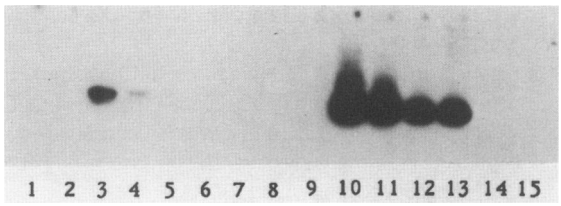

Figure 2. *PCR Amplification of HCV NT positive and negative strand RNA in serum. HCV positive serum was tested undiluted and at dilutions 1:20 and 1:1000 for positive strand (genomic) and negative strand (replicative) RNAs using NT antisense and sense primers, respectively, for reverse transcription followed by single (lanes 2–8) and double PCR (lanes 9–15). The PCR products were hybridized with end-labeled oligomer. Lane 1: Lambda DNA marker. Lane 2: Negative control for first PCR. Lane 3: Positive strand HCV RNA of undiluted serum. Lane 4: Positive strand HCV RNA of serum diluted 1:20. Lane 5: Positive strand HCV RNA of serum diluted 1:1000. Lane 6: Negative strand HCV RNA of undiluted serum. Lane 7: Negative strand HCV RNA of serum diluted 1:20. Lane 8: Negative strand HCV RNA of serum diluted 1:1000. Lane 9: Negative control for second PCR. Lane 10: Positive strand HCV RNA of undiluted serum. Lane 11: Positive strand HCV RNA of serum diluted 1:20. Lane 12: Positive strand HCV RNA of serum diluted 1:1000. Lane 13: Negative strand HCV RNA of undiluted serum. Lane 14: Negative strand HCV RNA of serum diluted 1:20. Lane 15: Negative strand HCV RNA of serum diluted 1:1000.*

times less than the positive strand in the tissues of two patients and the same in the third (Table 4). In serum of two of these patients, negative strand RNA was not detectable whereas positive strand RNA was present, in one case at high dilution (Table 4). These findings indicate that HCV RNA in liver or tumor extracts is not derived from blood contaminating the tissues. Furthermore, the data suggest that HCV may replicate in hepatocytes as well as in hepatocellular carcinoma cells, although we cannot exclude the possibility that HCV may replicate in non-parenchymal cells. A recent study however, using *in situ* hybridization for localization of HCV RNA in two chimpanzee livers demonstrated HCV RNA only in hepatocytes and not in Kupffer cells.[31]

The demonstration of HCV sequences in the majority of HCC tissues of anti-HCV positive patients supports the hypothesis that HCV may be associated with hepatocarcinogenesis. However, we cannot exclude the possibility that HCV, although present in the tumor cells, does not play any role in malignant transformation. Unlike retroviruses, HCV does not replicate by reverse transcription and no DNA intermediates of HCV have been detected.[24] It is more likely that HCV, rather than directly transforming hepatocytes, has an indirect effect on cell growth or differentiation such as activation of growth factors, oncogenes, or DNA binding proteins.

Table 4. *Comparison of Titers of Both Strands of HCV RNA (NT Sequences) in Liver and Tumor Tissues of Three Representative Cases*

Case no.		Positive strand titer	Negative strand titer
1	Liver	10^{-1}	10^{-1}
	Tumor	10^{-1}	10^{-1}
	Serum	10^{-0}	Not detected
3	Liver	10^{-2}	10^{-1}
	Tumor	10^{-2}	10^{-1}
	Serum	10^{-4}	Not detected
10	Liver	10^{-3}	10^{-2}
	Tumor	10^{-2}	10^{-1}

There was a good correlation between the presence of anti-HCV in serum and HCV sequences in liver ($P < 0.025$). Only one anti-HCV positive patient contained no demonstrable HCV sequences in liver or tumor tissues. However, 4 of 12 anti-HCV negative patients contained HCV NT sequences in the liver and/or tumor with one of these positive for NS3 sequences in both liver and tumor tissues. These findings support the observation that the first-generation anti-HCV assay (anti-c100-3) used in this study may result in false-positive or false-negative reactions.[14–18]

X gene sequences of HBV were detectable in 5 of 12 HCV-negative patients in whom tissues were available for testing by PCR. One patient had both HCV sequences and HBV X sequences in the tumor tissue. Interestingly, in three of these cases, HBV X sequences were only found in the liver tissues. This may be related to mutations or deletions of this region of the HBV genome when integrated into the DNA of tumor cells. PCR results for HBV S gene sequences were identical except for the liver tissue of case 14 in which S gene sequences were not detectable although X gene sequences were present. We have reported similar findings.[32] In two of the five cases with HBV X sequences, no markers of current or past HBV infection were detected in the serum suggesting that HBV sequences may persist in liver tissues even after elimination of all viral markers from the serum.[25,33]

Finally, in this series of unselected cases, only three patients were without any evidence of HBV or HCV infection using very sensitive PCR methodology. No known risk factors for HCC other than cirrhosis in two cases were elicited in these patients. Although the etiology of HCC appears to be multi-factorial, these findings suggest that the majority of HCC in the United States may be related to viral infections.

Acknowledgments

We thank Mrs. Leslie Wulfekuhler and Ms. Teri Heidel for expert secretarial assistance.

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