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Hepatitis C virus RNA in southern African Blacks with hepatocellular carcinoma

(non-A, non-B hepatitis/chronic hepatitis/polymerase chain reaction)

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ABSTRACT **We used a sensitive and specific cDNA polymerase chain reaction assay to detect hepatitis C virus (HCV) RNA in serum samples from 128 unselected southern African Blacks with hepatocellular carcinoma (HCC) and found HCV RNA in 26 (20.3%) of these patients. Antibodies to HCV (anti-HCV) were detected in 59 patients (46.1%) with a first-generation ELISA test, and only 19 of these patients were HCV RNA-positive. Anti-HCV was detected in 25 patients (19.5%) with a second-generation ELISA test, and 17 of these patients were HCV RNA-positive. Among the second-generation ELISA- and HCV RNA-positive patients, 14 were anti-HCV positive, 2 were indeterminate, and 1 was anti-HCV negative in a second-generation recombinant immunoblot assay, whereas all patients who were second-generation ELISA positive, but HCV RNA negative, were anti-HCV negative in this immunoblot assay. A total of 5 patients were negative in both ELISA tests but were HCV RNA positive. Seventy-one patients (55.5%) had evidence of a current HBV infection, 50 (39.1%) had evidence of a previous infection, and 7 (5.5%) had no evidence of a current or previous HBV infection. The prevalence of current HBV infection was significantly lower in patients who were positive for HCV RNA than in those who were negative ($P = 0.001$). This difference was not dependent on sex, age, or geographical location of the patients. The mean age of HCC patients positive for HCV RNA (52.3 years) was significantly higher ($P < 0.001$) than that of negative patients (40.3 years), and the difference was not dependent on HBV status or geographical location. Patients positive for HCV RNA were more likely to be urban than were negative patients. Thus, a significant number of southern African Blacks with HCC had a current HCV infection but not a current HBV infection, further suggesting that infection with HCV plays a role, albeit minor, in the development of HCC in this population.**

Hepatitis C virus (HCV), the etiological agent of most parenterally transmitted non-A, non-B hepatitis (1–3), is a major cause of chronic hepatitis and liver cirrhosis (4) and, possibly, hepatocellular carcinoma (HCC) (5–8). Numerous studies have documented an association between the presence of antibodies to HCV (anti-HCV) and development of HCC in the presence or absence of coinfection with hepatitis B virus (HBV; refs. 9–33). Some of these studies have found HCV and HBV to be cofactors in the development of HCC, whereas others have found HCV and HBV to function independently. However, serologic tests suffer from problems of nonspecificity and are not reliable indicators of current HCV replication. Reverse transcription of HCV RNA, followed by PCR amplification of the cDNA, is currently the best method to demonstrate HCV viremia (3, 34–36). We examined sera from 128 southern African Blacks with HCC for the presence of HCV RNA by PCR to determine whether HCV infection played a significant role in the development of HCC.

PATIENTS AND METHODS

Included in this study were 128 unselected southern African Blacks (112 males, 16 females) with biopsy-verified HCC. The mean age of the 116 patients whose age was known was 42.9 years (range, 17–80 years). The geographical location of the patients was as follows: 56 were born in, and had always lived in, a rural location ("rural"); 52 were born in a rural area but had migrated to an urban location as young adults ("rural–urban"); and 12 patients were born in, and had always lived in, an urban location ("urban"). For 8 patients the geographical location was unknown. Seventy-one patients (55.5%) had evidence of current HBV infection [positive for serum HBV-encoded surface antigen (HBsAg) and HBV core antibodies (anti-HBc)], and 50 patients (39.1%) had evidence of previous infection [positive for serum HBV surface antibodies (anti-HBs) and/or anti-HBc]. Seven patients (5.5%) lacked all serological markers of HBV infection.

All tests were performed on a single serum sample that was collected between 1987 and 1990 and stored at −20°C to −80°C until testing. All sera were tested for anti-HCV with first-generation (Ortho Diagnostic) and second-generation (Abbott) ELISA tests (2). In addition, we tested sera that were positive in the second-generation ELISA test with a second-generation recombinant immunoblot assay (RIBA) for antibody to three HCV antigens (RIBA HCV test system, Chiron). The results of the three anti-HCV tests were interpreted according to the instructions of the manufacturer. HBV markers were measured by radioimmunoassay (Abbott).

The "nested" cDNA PCR assay was used to detect HCV RNA in 100 μl of serum, as described previously (36). The primer set used in our assay (primer set *a* in ref. 36) is specific for two nucleotide domains within the 5′ noncoding region of the HCV genome that we have shown to be highly conserved among HCV isolates (37). Furthermore, we have demonstrated that this primer set is highly effective in detecting HCV RNA in samples collected worldwide (36). A 10-μl aliquot of the PCR product from the second round of amplification was analyzed by electrophoresis in a 2% agarose gel, followed by staining with ethidium bromide and visualization under ultraviolet light. Specificity of DNA bands was confirmed by high-stringency Southern blot hybridization (36). To reduce the risk of contamination in the PCR analysis, a number of standard precautions were taken (36) and negative serum controls were interspersed and analyzed in parallel with the test samples to monitor for contamination as a source of false positive results. We did not have any false

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; anti-HCV, antibodies to HCV; HBV, hepatitis B virus; RIBA, recombinant immunoblot assay.

positive results among 120 negative serum controls tested. The sensitivity of our nested cDNA PCR assay, previously shown to be <1 chimpanzee infectious dose, was monitored throughout the analysis by including a positive control consisting of a 10^{-6} dilution of HCV reference strain H that was at the limit of detection of the assay (36); all 28 such samples were HCV RNA positive.

The data were analyzed statistically by several methods. The mean ages of two groups of subjects (e.g., HCV RNA-positive vs. HCV RNA-negative patients) were compared by Student's *t* test (ref. 38, pages 114–119). Interactions (ref. 38, pages 175–181) between ages classified according to HCV RNA status, HBsAg status, and geographical status were not significant. Association between a pair of attributes (e.g., sex vs. HCV RNA positivity) was tested by comparing the corresponding relative frequencies in a 2-fold table using a χ^2 statistic (ref. 38, pages 242–243); strength of association was estimated with a Kendall *V* statistic (39). Interaction in classifications with three attributes was assessed by the Gart–Thomas method (40). χ^2 statistics with 2 df were partitioned as described by Cochran (41).

RESULTS

HCV RNA was detected in 26 (20.3%) of 128 unselected southern African Blacks with HCC (Fig. 1). Anti-HCV were detected in 59 patients (46.1%) with the first-generation ELISA test and in 25 patients (19.5%) with the second-generation ELISA test (Table 1). There was a significant correlation between the presence of HCV RNA and anti-HCV measured with the second-generation ELISA ($P < 0.001$), as well as with the first-generation ELISA ($P < 0.01$). Among the 17 patients positive by second-generation ELISA and PCR, 14 were positive, 2 indeterminate (both with 4+ response against the c22-3 antigen), and 1 negative when tested with RIBA, whereas all 8 patients who were second-generation ELISA positive, but HCV RNA negative, were anti-HCV negative in the RIBA (Table 2). It is noteworthy that 6 of the RIBA-negative patients had at least one visible antigen band of intensity less than the lower positive control band, suggesting that anti-HCV were present but at very low titer. The RIBA-negative patients had significantly lower

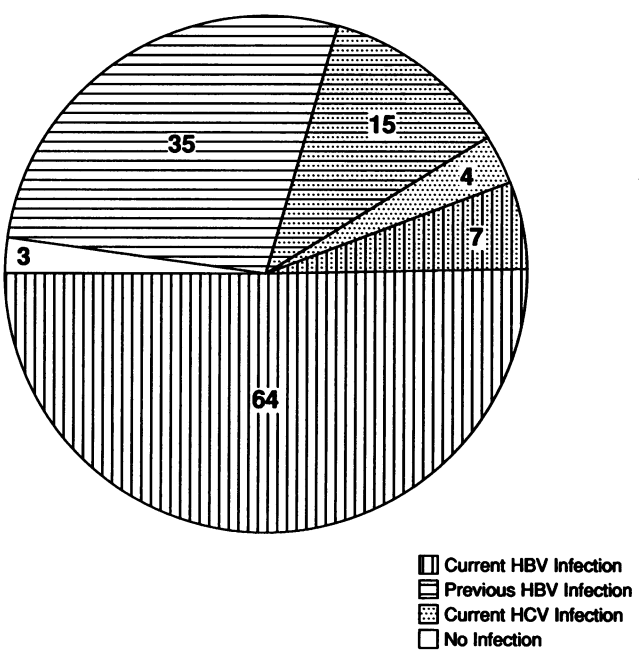

FIG. 1. HBV and HCV status in 128 southern African Blacks with HCC. Number of patients in each category is indicated.

Table 1. Patient HCV antibody and RNA status

ELISA		No. of patients (%)		
First generation	Second generation	Total	HCV RNA positive	HCV RNA negative
Negative	Negative	65	5 (8)	60 (92)
Positive	Negative	38	4 (11)	34 (89)
Negative	Positive	4	2 (50)	2 (50)
Positive	Positive	21	15 (71)	6 (29)

optical density values in the second-generation ELISA than the RIBA-positive and RIBA-indeterminate patients, suggesting that the RIBA test was less sensitive than the second-generation ELISA.

We also examined the relationships between HCV infection and HBV infection, mean age, geographical location, and sex (Table 3). The prevalence of current HBV infection was significantly lower in patients positive for HCV RNA than in negative patients ($P = 0.001$, $V = -0.30$). This negative correlation was not dependent on sex, age, or geographical location ($3 \times 2 \times 2$ contingency table test for interaction). The mean age of HCC patients positive for HCV RNA (52.3 years) was significantly higher than that of negative patients (40.3 years; $P < 0.001$), and the difference was not dependent on HBV status or geographical location. Conversely, the mean age of HCC patients with markers of current HBV infection (37.0 years) was significantly lower than the mean age of patients with markers of previous HBV infection or patients with no HBV markers (49.6 years; $P < 0.001$), and this difference was not dependent on HCV status or geographical location. A significantly higher proportion of urban residents was found among the patients positive for HCV RNA than among the negative patients ($P < 0.02$). Conversely, there was a significantly lower proportion of urban residents among patients with evidence of current HBV infection than among patients with evidence of previous or no HBV infection ($P < 0.001$). There was no significant difference in the sex of patients positive or negative for HCV RNA. In contrast, patients with current HBV infection were significantly more likely to be males than patients without current HBV infection ($P < 0.01$).

DISCUSSION

Almost all previously published reports of the role of HCV in the development of HCC have been based on an association with anti-HCV. The purpose of this study was to determine whether first- and second-generation ELISA tests for anti-HCV were valid indicators of current HCV infection in southern African Blacks with HCC and whether such infection might be an independent factor in the etiology of HCC. We used a nested cDNA PCR assay to detect HCV RNA in the serum of 128 unselected southern African Blacks with HCC. To ensure optimal sensitivity, we demonstrated that the nested cDNA PCR assay could detect minute amounts of HCV RNA under standard conditions. A positive control containing HCV RNA at the limit of detection was included in each extraction series, and 120 negative serum controls were interspersed and tested in parallel with the experimental

Table 2. RIBA status in 25 second-generation ELISA-positive patients

Second-generation RIBA	No. of patients (%)		
	Total	HCV RNA positive	HCV RNA negative
Positive	14	14 (100)	0
Indeterminate	2	2 (100)	0
Negative	9	1 (11)	8 (89)

Table 3. Patient characteristics according to HCV RNA status

	HCV RNA positive (n = 26, 20.3%)	HCV RNA negative (n = 102, 79.7%)
HBV infection		
Current	7 (26.9%)*	64 (62.7%)
Previous	15 (57.7%)	35 (34.3%)
Neither	4 (15.4%)	3 (2.9%)
Age, years		
Range	27–78	17–80
Mean	52.3 (n = 25)†	40.3 (n = 91)
Geography		
Rural	7 (26.9%)	49 (52.1%)
Rural–urban	13 (50.0%)	39 (41.5%)
Urban	6 (23.1%)‡	6 (6.4%)
Unknown	0	8
Sex		
M/F ratio	21:5 (80.8% M)	91:11 (89.2% M)

P value refers to comparison of presence of HCV RNA.
*$P = 0.001$, $V = -0.30$ (negative correlation independent of age, sex and geographical location).
†$P < 0.001$ (difference independent of HBV status and geographic location).
‡$P < 0.02$.

samples without contamination. The specificity of the PCR amplification products was confirmed by high-stringency Southern blot hybridization. Finally, we used sera that were collected recently and stored under ideal conditions. Thus, we believe that our results in the cDNA PCR assay are reliable in identifying HCV viremia.

HCV RNA was detected in 26 (20.3%) of 128 southern African Blacks with HCC. We detected HCV RNA in only 19 of 59 HCC patients who were anti-HCV positive in a first-generation ELISA and in 17 of 25 HCC patients who were anti-HCV positive in a second-generation ELISA test. The lack of detection of HCV RNA in anti-HCV-positive HCC patients can result from a level of HCV below the detection limit of our PCR assay. Alternatively, the presence of anti-HCV in these cases might reflect a previous HCV infection or result from nonspecificity of the tests. Eight percent of patients who were anti-HCV negative in both ELISA tests were HCV RNA positive. We conclude that, even though we found a significant positive correlation between the presence of HCV RNA and the results of first- and second-generation ELISAs in HCC patients, currently available antibody tests do not accurately reflect the HCV status of the patients tested.

Although it is not required for the interpretation of the Abbott second-generation ELISA, the serum of positive patients was tested with the second-generation RIBA test. Interestingly, all 8 ELISA-positive but HCV RNA-negative patients were RIBA negative, whereas 14 of the 17 ELISA- and HCV RNA-positive patients were RIBA positive. Our data indicate that this reflects a lower sensitivity of the RIBA test compared with the second-generation ELISA (see *Results*), and it is conceivable that patients with a previous HCV infection would have lower levels of anti-HCV than patients with ongoing HCV infection. Our data further indicate that RIBA-indeterminate results based on a single positive reaction, usually to the c22-3 antigen band, most likely should be considered positive. The interpretation of results by the second-generation RIBA test needs to be addressed further.

We previously demonstrated that a significantly higher proportion of HCC patients than of matched controls were positive for anti-HCV among southern African Blacks (11). Similar differences have been observed in Europe, the United States, and Asia (9, 13–15, 20–22, 25–27, 29, 30, 32, 33). In this study we have demonstrated that a substantial number of anti-HCV-positive HCC patients among southern African Blacks have actual HCV infection. Taken together, these data clearly prove an association between HCV infection and HCC.

Other groups have studied small numbers of HCC patients and have found that HCV RNA persists in the liver tissue and the serum of some of these patients (42–44). In our study of a large group of unselected southern African Blacks with HCC, HCV RNA was detected in 20.3%. Interestingly, our statistical analysis revealed a strong negative correlation between a current HBV infection and the presence of HCV RNA. Furthermore, the characteristics of patients positive for HCV RNA differed from those of patients with evidence for current HBV infection. Thus, although HBV may be the dominant etiological agent responsible for HCC among southern African patients, HCV infection appears to play an important role in the cases not directly related to chronic infection with HBV. Recently, Ruiz *et al.* (45) reported the finding of HCV RNA in 62% of sera from 68 Spanish HCC patients. Their data are in agreement with our finding that HCV plays an independent role in the development of HCC, but in contrast to our finding in southern African HCC patients, their data suggest that HCV plays a more important role than HBV in the development of HCC in Spain. These contrasting studies strongly suggest the existence of marked geographical differences in the etiology of HCC. Similarly, it is noteworthy that we find HCV infection to be more frequent among urban residents than among rural residents, in contrast to current HBV infection, which has the opposite distribution. Thus, HBV and HCV appear to have different etiological roles in the development of HCC in different countries and even in different regions of the same country.

Despite evidence for an etiological role of HBV and HCV in the development of HCC, the mechanism by which infection leads to HCC is not fully understood. The higher mean age of HCC patients positive for HCV RNA in this study suggests (*a*) a different carcinogenic mechanism in HCV-related cases than in HBV-related cases, (*b*) a different degree of virulence or carcinogenicity, or (*c*) infection at a later age. At present, it is not possible to differentiate among these. Nevertheless, regardless of mechanism, HCV appears to have a small but significant and independent role in the development of HCC in southern African Blacks.

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