HEPATOCELLULAR CARCINOMA IN EGYPTIANS WITH AND WITHOUT A HISTORY OF HEPATITIS B VIRUS INFECTION: ASSOCIATION WITH HEPATITIS C VIRUS (HCV) INFECTION BUT NOT WITH HCV RNA LEVEL

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Abstract. The aim of this study was to analyze the association of hepatocellular carcinoma (HCC) with hepatitis C virus (HCV) in Egypt, using hepatitis B virus (HBV) and hepatitis E virus (HEV) as virus controls. In addition, the association of HCC with HCV RNA levels among persons seropositive for HCV was analyzed. We compared 131 patients with proven HCC, 247 with bladder cancer, and 466 healthy hospital employees. Age, sex, and place of residence were recorded to study confounding factors. Among the healthy controls, 16% were seropositive for HCV, 21% for HBV, and 31% for HEV. When healthy controls were age-matched with HCC patients, the latter were significantly (P < 0.001) more often HCV seropositive (67%) than were the controls (30%). The seropositivity for HBV and HEV did not differ significantly in frequency between the two groups. The seropositivity for HCV was also significantly (P < 0.001) more often found in HCC patients (76%) than in BC patients (47%), with seroprevalences for HBV and HEV not differing significantly in these age-matched groups. In HBV-negative HCC and bladder cancer patients, seroprevalence for HCV was significantly (P = 0.002) higher in HCC patients (68%) than in bladder cancer patients (36%). This difference was even more pronounced (P < 0.001) in HBV-positive HCC and bladder cancer patients (78% versus 52%, respectively). Of HCV-seropositive individuals, 49% were HCV RNA positive by branched DNA assay, and of these, 96% were infected by HCV genotype 4. No correlation between HCV RNA load and seropositivity of HBV or age or disease state was found. Infection with HCV and HCV-HBV double infection, but not HBV or HEV infection alone, is strongly correlated with HCC in Egypt.

Hepatitis C virus (HCV) causes more than 90% of parenterally transmitted non-A, non-B (NANBH) hepatitis.1–4 In more than 85% of HCV infections, the infection persists and leads to chronic hepatitis.4,5 Once chronic hepatitis is established, it may slowly progress to worsening stages of fibrosis and cirrhosis and ultimately lead to the development of hepatocellular carcinoma (HCC).6–8 In the first two decades after acute HCV infection, both mortality and morbidity remain modest in frequency, but after this period both can be expected to increase.1,2 Infection with HCV is widely recognized as the most common indication for liver transplantation9 in the United States, Europe, and Japan.

Although no firm data are available, death rates for HCC in Egypt appear to be increasing over the last decade.10,11 Hepatitis virus infections of any kind are highly prevalent in the general population. The relatively high prevalence of HCV infections in Egypt was first established in 1992 by Kamel and others.12 Other groups thereafter found HCV seroprevalence rates ranging from 6% to 38% with an average of approximately 15%.13–16 These results suggest that the current HCV epidemic was at least partially caused by parenteral treatment with praziquantel about 10 years ago.17,18 Such therapy is currently the major route of HCV spread in Egypt.

It has been suggested that although schistosomiasis is not associated with HCV infection,19 a relationship exists between HCV infection and parenteral treatment for schistosomiasis.20–22 This relationship does not appear to exist for hepatitis B virus (HBV).23 Schistosomiasis, a major risk factor for the development of bladder cancer,24 is hyperendemic in Egypt, and the death rates for bladder cancer are among the highest in the world.25 According to Kamel and others, Schistosoma mansoni infection is typically present in 50–60% of people 10 years of age and older living in rural areas of the Nile delta.19 The mean age of patients with schistosomiasis-related bladder cancer was 46 years and that of patients with bladder cancer without a relationship with schistosomiasis was 63 years.11 Severe hepatic fibrosis, a serious complication of schistosomiasis, has a peak incidence in persons 40–60 years of age, the same age group in which the prevalence of HCV is highest. Some hospital-based studies have found a relationship between schistosomiasis and HCV infection in Egypt22,26,27 that is probably confounded by age and sex. Yet, careful population-based studies failed to show a relationship between schistosomiasis and HCV or HBV infection.19 However, it still remains possible that the current HCV epidemic was at least partially caused by parenteral treatment with tarter emetic injections for schistosomiasis, as has been suggested by others.20–22 Large national campaigns for parenteral treatment for schistosomiasis started approximately 40 years ago and have been abandoned for oral treatment with praziquantel about 10 years ago.29 Only large prospective studies can elucidate the role of past parenteral treatment for schistosomiasis in the present HCV epidemic in Egypt.

Hepatitis B virus is also hyperendemic in Egypt, with seroprevalence rates ranging from 24% in the general population16 to 66% in persons 40–67 years of age.29 It has been reported that HCV and HBV have a reciprocal inhibitory
effect on each other’s replication levels.\textsuperscript{11,30,31} Coinfection with HBV is believed to lead to an aggravated course of disease, and faster progression to HCC.\textsuperscript{32,33} Alcohol abuse also may aggravate the course of HCV infection.\textsuperscript{34–36} However, although alcohol consumption is not uncommon in Egypt,\textsuperscript{37} overt alcoholism is rare and would have only marginal influence on the natural history of hepatitis infections.

The aim of this study was to analyze the association of HCC with HCV, using HBV and hepatitis E virus (HEV) as virus controls, in an HCV hyperendemic area with a high prevalence of HCC. Hepatitis E virus is an enterally transmitted RNA virus causing acute but not chronic hepatitis and assumed not to be implicated in the development of HCC.\textsuperscript{38,39} The association of HCC with HCV in relation to confounding factors such as age, sex, and geography was analyzed in a large cohort of Egyptian patients with histologically proven HCC, using patients with bladder cancer and disease free hospital employees as controls. These three populations were compared for seroprevalence of HBV, HCV, and HEV. Samples positive for HCV antibody were analyzed for RNA copy numbers and HCV genotype by the branched DNA (bDNA) technique and the line probe assay, respectively.

MATERIALS AND METHODS

The study population included 131 patients with HCC, 247 patients with bladder cancer, and 466 healthy controls. They were recruited among hospital patients and employees of the National Cancer Institute (NCI) of the University of Cairo. Participation in the study was voluntary, and after extensive information was given to either the participants or their parents, informed consent was obtained from all subjects. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the Amsterdam Academic Medical Center Ethical Board. Blood samples for serologic and virologic analyses were taken and participants were interviewed using a standardized questionnaire that included questions about clinical symptoms and medical history.

Diagnosis of HCC was based on clinical and laboratory findings as well as histopathologic features of the liver as defined by the Edmondson-Steiner grading system.\textsuperscript{40} The diagnosis of bladder cancer was based on clinical characteristics.\textsuperscript{11} Healthy controls were recruited from NCI personnel, including civil servants, nurses, laboratory technicians, doctors, students, and children from NCI personnel, all of whom had no symptoms or clinical signs of liver disease.

Serologic markers for HBV (hepatitis B surface [HBs] antigen, and antibodies to hepatitis B core antigen [anti-HBc]) and HEV (antibodies to HEV) infection were detected with current standard assays (enzyme immunoassay [EIA]; Abbott Laboratories, Abbott Park, IL). A sample was considered positive for HBV when found positive for HBs antigen or anti-HBc antibodies, or both. Antibodies to HCV were detected with HCV EIA version 3.0 (Abbott Laboratories). All serologic assays were carried out according to the manufacturer’s instructions.

Serum HCV RNA copy numbers were determined by an HCV quantitative bDNA assay with a detection limit of $10^{3.3}$ copies/ml. Mean copy numbers were calculated by assigning bDNA negative samples a value of half the cut-off value. Reagents were kindly provided by the Chiron Corp. (Emeryville, CA). Analysis of HCV genotypes was done with the line probe assay (LIPA) (Innogenetics NV; Antwerp, Belgium) and performed according to the manufacturer’s instructions.

Statistics were calculated using SPSS (SPSS Institute, Chicago, IL) for DOS software. The Mann-Whitney test was used to compare groups.

RESULTS

Study population characteristics. Table 1 shows the epidemiologic, clinical, and laboratory data for the 844 individuals in the study population. Alanine aminotransferase (ALT) values were assessed in all sera. Age, sex, and place of residence were well matched between patients with HCC and bladder cancer. The mean ± SD ages in those groups were 55 ± 12 years versus 56 ± 10 years, respectively, but the mean age of healthy controls (20 ± 15 years) was significantly lower compared with the cancer groups (Table 1). The majority (n = 289, 61%) of the healthy controls were 0–24 years of age, whereas fewer (n = 11, 2%) healthy controls were 50 years of age and older.

Since age-matching was needed to exclude age as a confounding factor for HCC, statistical analyses of virologic differences between the healthy subjects and those with HCC were performed by analyzing virologic data in an age-matched subpopulation. A 18–63 years age stratum of the two groups was narrowed at the upper and lower limit until the mean ages of the groups were no longer significantly different. This resulted in an age-matched stratum of patients between 41 and 53 years. Within this stratum, healthy individuals had an mean ± SD age of 47 ± 3 years whereas patients with HCC had a mean ± SD age of 48 ± 3 years, and sex and place of residence were also well matched.

Seroprevalence of HCV, HBV, and HEV in relation to age in healthy controls. The age distribution of hepatitis virus infection was studied in healthy controls (Table 2). Healthy controls were divided into four age groups: 0–2, 3–20, 21–35, and > 35 years of age. The male: female ratio was approximately equal in all age groups. The seropreva-
Seroprevalence of HBV, HCV, and HEV in relation to age in cancer patients. The bladder cancer and HCC patients were divided into three age groups: (20–50, 51–65, and > 65 years of age). (Table 3). The male:female ratio was approximately equal in all age groups. Statistical analysis of these data showed that hepatitis virus seroprevalences did not differ significantly with age within the study groups (Table 3). For example, in HCC patients, the seroprevalence of HCV was approximately 75% in all age groups, while in bladder cancer patients, the seroprevalence of HCV was approximately 50% in all age groups. The same held true for HBV and HEV. The differences in seroprevalences between study groups were analyzed separately.

Sex distribution of HCV, HBV, and HEV in the study population. Infections with HCV and HEV were equally distributed between males and females. However, more (P < 0.001) HBV infections were found in males. When clinical status was taken into account, significantly more HCV and HBV infections were found in HCC patients and significantly more were present in males compared with females (82% and 84% versus 61% and 51%, respectively). Such a difference was not found in the patients with bladder cancer (45% and 74% versus 54% and 60%, respectively) and healthy controls groups (18% and 23% versus 14% and 19%, respectively). No significant differences were found between any groups concerning HEV infections.

Seroprevalence of HBV, HCV, and HEV in HCC patients versus healthy controls and HCC patients versus patients with bladder cancer in age-matched strata. The seroprevalence of HCV, HBV, HCV-HBV, and HEV infections in the healthy controls versus the HCC groups were analyzed in the age-matched population of > 41 and < 53 years of age. Patients with HCC had a significantly (P < 0.001) higher seroprevalence of HCV infection (67%) than the healthy control group (30%). Seroprevalence of HBV (66% in the HCC group versus 46% in the controls) and HEV (49% versus 38%) were not significantly different in these age-matched groups (Figure 1A).

When hepatitis virus seroprevalence in the HCC and bladder cancer patient groups was analyzed (Figure 1B), infection with HCV was significantly (P < 0.001) higher in the HCC group than in the bladder cancer group (76% versus 47%). Seroprevalence of HBV (74% in the HCC group versus 61% in the bladder cancer group) and HEV (51% versus 50%) did not differ significantly. These results indicate that HCV infection and not HBV infection alone is correlated with HCC.

Seroprevalence of HCV and HEV in HCC patients compared with bladder cancer patients with and without HBV infection. The significance of HBV coinfection was...
PATIENTS; blank columns of 288 HCV-seropositive individuals were either negative for HCV RNA or had a load less than $10^6$ copies/ml. Thus, 230 (80%) had a load at least $10^6$ copies/ml. Therefore, 146 (51%) had an HCV RNA load below the detection limit, and 84 (29%) of 288 had an HCV RNA load between detection limit and $10^6$ copies/ml. Thus, 230 (80%) of 288 HCV-seropositive individuals were either negative for HCV RNA or had a load less than $10^6$ copies/ml.

Age did not correlate with the RNA load. No significant difference among the study groups was found in the percentage of HCV-seropositive samples negative in the bDNA assay. The mean $\pm$ SD HCV copy number in HCC patients was $10^{6.73} \pm 0.47$ compared with $10^{5.60} \pm 0.43$ in bladder cancer patients and $10^{5.70} \pm 0.51$ in healthy controls. Since it has been reported that HBV may reduce HCV replication and vice versa, HCV RNA copy numbers were analyzed in both HBV+ and HBV- individuals. The mean HCV RNA load in HBV+ individuals ($10^{6.69}$ copies/ml) was not significantly different from the RNA load in HBV- individuals ($10^{6.66}$); also no difference was seen when the clinical status of the patients was taken into account.

**Genotype of HCV.** Genotyping of HCV was attempted for 89 randomly selected HCV RNA-positive samples. Twelve samples could not be genotyped, probably because of low RNA copy numbers, and in the other 77 samples a genotype could be determined. Seventy-four samples (96%) had genotype 4. Four samples (5.2%) showed cross-reactivity with genotype 1a, which is probably indicative of double infections. Two samples (2.6%) had an HCV strain genotype 1 (subtype 1a) and one (1.3%) had genotype 3a.

**DISCUSSION**

The high baseline prevalence of infection with hepatitis viruses in Egypt makes it difficult to establish a disease association. For example, HCV seroprevalence increases with age to about 40% in healthy people in their forth and fifth decades. After middle age (35–45 years old), the HCV seroprevalence stabilizes at this level. This was seen both in the present study and in studies by others. Although blood banks in Egypt started to screen for HCV in 1993, blood transfusion is still a major risk factor for acquiring HCV infection, since blood donated for transfusion is commonly supplied by relatives of patients rather than blood banks. Surgical treatment with contaminated equipment may represent another major risk factor, because all hospitals may not disinfect their equipment properly. Other common risk factors for acquisition of HCV infection, such as parenteral drug abuse, are very uncommon in Egypt. In the present study, this has been taken into account by including the bladder cancer patient group, which has characteristics similar to the HCC group concerning hospital visits and surgical treatment.

Based on the data in this paper, it is apparent that HCV and HBV infections are acquired in adolescence or early adulthood ($> 20$ years old), or that most infections occurred at least 20 years ago. Infection with HCV and coinfection with HBV but not HBV or HEV infection alone were correlated with HCC. Also, HCC was strongly related to HCV infection irrespective of HBV or HEV infection. This is in agreement with other reports which showed a stronger association of HCC with HCV and HCV-HBV double infection than with HBV infection alone. Infection with HCV was present in 76% of the HCC patients, which is higher than the 65% found by Waked and others. Thus, 24% of the HCC remains unexplained. If it is assumed that HCV or HBV can independently cause HCC, only 8% of cases remain unexplained. This suggests that while HCV is not the only factor in HCC development in Egypt, this particular virus is a major risk.

Several cohort studies on the natural history of HCV have
shown that 15–33% of the individuals chronically infected with HCV may develop HCC 20–30 years after acute HCV infection. When the impact of age on the acquisition of HCV infection in the present study was investigated, HCC appeared to develop 2–3 decades later than when HCV infection is generally acquired. Taken together with the finding that HCV is mainly acquired after the age of 20, these data are consistent with those of previous reports.

Despite the fact that HCV genotype 4 is sometimes found in Europe, this genotype is the predominant one in the Middle East and Africa, excluding the Republic of South Africa. In this study, 96% (74 of 77) of the Egyptian HCV strains were genotype 4. This is consistent with the report by McOmish and others, who found genotype 4 to be the predominant one in Egypt. Parallels may be drawn between the nature of HCV and human immunodeficiency virus (HIV) infection because of similarities in genetic diversity and level of viral load. High HIV RNA copy numbers are associated with more rapid progression to acquired immunodeficiency syndrome (AIDS), whereas low copy numbers are associated with delayed development of AIDS. However, it has recently become apparent that it is unlikely that a similar relationship exists for HCV concerning viral load. We investigated whether HCV RNA copy numbers as measured by bDNA were associated with disease status defined by the presence of HCC. The mean HCV RNA copy number for all HCV-positive samples was relatively low, 10^4–10^5/ml of serum, and no significant differences were seen between study groups. Others have also found particularly low HCV RNA copy numbers in patients infected with HCV genotype 4. Together with the current results, this suggests that HCV genotype 4 generally has lower RNA loads compared with genotypes 1–3. If this proves to be true, it could mean that future trials for HCV therapy in Egypt will be difficult to monitor using current techniques, given their sensitivity and/or their inability (with the exception of the bDNA assay) to quantify all HCV genotypes.

There was no significant difference in mean HCV RNA load in HBV-positive and HBV-negative samples, indicating that in this setting, HBV infection does not have an impact on HCV load. Zarski and others showed that in HBsAg- and HBV DNA-positive patients, HBV double infection lowers HCV RNA load. Others have suggested that HCV and HBV have a reciprocal inhibitory effect on each other’s replication. A possible explanation for the absence of any impact of HBV on HCV RNA levels is that the present study did not focus on active HBV replication as measured by HBs-antigen testing and only HBc antibody-positive serum samples were analyzed.

No significant differences in HCV RNA loads were observed between the study groups and controls. This finding, combined with the fact that ALT values were low and not clearly linked to HCV infection or HCC in this study, confirmed earlier findings. The observation that HCV infection rather than the level of replication was associated with HCC, suggests that in the case of HCV it is not the viral load that is a determinant of disease progression, but rather the immune response raised against HCV. Khakoo and others reported that the hepatic injury found in patients with chronic hepatitis C may be induced by cytotoxic T cell responses against infected liver cells. In this view, this continuing process of hepatic damage might eventually lead to the development of HCC.

In conclusion, our study shows that HCC in Egypt is strongly associated with HCV infection, particularly in the presence of an HBV coinfection, although HBV infection status did not influence the serum HCV RNA load related to HCC. The HCV RNA load was relatively low in all individuals, of whom the vast majority were infected with HCV genotype 4.

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