CASE-CONTROL STUDY ON HEPATITIS C VIRUS (HCV) AS A RISK FACTOR FOR HEPATOCELLULAR CARCINOMA: THE ROLE OF HCV GENOTYPES AND THE SYNERGISM WITH HEPATITIS B VIRUS AND ALCOHOL

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We performed a case-control study to evaluate the risk of hepatocellular carcinoma (HCC) for hepatitis C virus (HCV) infection. A total of 305 newly diagnosed HCC cases (80% males) and 610 subjects (81% males) unaffected by clinically evident hepatic disease admitted to the main hospitals in Brescia, North Italy, were recruited as cases and controls, respectively. Among the 122 HCC cases positive for HCV RNA, genotype 1b was found in 83 patients (68%), genotype 2 in 36 (29.5%) and genotype 3a in 3 (2.5%). Among the controls, 15 were infected with genotype 1b and 15 with type 2. Analysis of HCV envelope 1 nucleotide sequence among 25 cases and 28 controls infected with genotype 2 showed subtype 2c in 96% of cases and in all controls, and subtype 2a in 1 HCC case. The odds ratio (OR) for HCV RNA positivity adjusted for hepatitis B virus (HBV) markers and alcohol intake was 26.3 (95% confidence interval: 15.8-44). It was higher for genotype 1b (OR = 34.2) than genotype 2 (OR = 14.4). The OR for HCV RNA was 35.6 (95% CI: 14.5-87.1) when the HBV markers were all negative and 132 (15.3-890) when HBsAg positivity was present; the OR was 26.1 (95% CI: 12.6-54.0) among subjects with alcohol intake of 0-40 g/day and increased to 62.6 (23.3-168) and 126 (42.8-373) with an alcohol intake of 41-80 and >80 g/day, respectively. In conclusion, synergism was found between HCV infection and HBV infection and alcohol intake in causing HCC. Int. J. Cancer 81:695-699, 1999.

Hepatitis C virus (HCV) has been definitely recognized as a major cause of chronic liver diseases, including hepatocellular carcinoma (HCC). However, the relative risk for HCC by HCV infection has been found to vary widely from one study to another (Donato et al., 1998). Various factors may promote progression of chronic liver disease toward HCC among subjects with virus-related, host-related and exogenous HCV chronic infection. Among these, HCV genotypes have been suggested to possess different potentials for evolution of the liver disease toward cirrhosis and HCC, but the results of studies carried out so far are conflicting (Brechot, 1997). Furthermore, the interaction of HCV infection with other known risk factors for HCC is poorly understood.

Our aim was to evaluate the risk of having HCC in individuals with HCV infection according to anti-HCV and HCV RNA status, HCV genotypes, markers of hepatitis B virus (HBV) infection and alcohol consumption through a case-control study enrolling a relatively high number of subjects in a high-incidence area for HCC in North Italy (Chiesa et al., 1995).

MATERIAL AND METHODS

Subjects

The study design has been previously described in full (Donato et al., 1997) and will only be summarized here. Inclusion criteria for HCC cases were being born in Italy, living in the province of Brescia for more than 5 years and admitted to the 2 main hospitals in the area in the study period. Of the 305 incident cases included, and hospitalized between January 1995 and March 1998, 259 (85%) were diagnosed by histology or cytology or had α-fetoprotein serum levels >500 ng/ml and the remaining 46 were diagnosed on the basis of sonography or computerized tomography. As controls we enrolled 610 subjects admitted to the Departments of Ophthalmology, Dermatology, Urology, Cardiology and Internal Medicine of the same hospitals and who were unaffected by liver disease or malignant neoplasm. The inclusion criteria used for HCC cases were also applied in the selection of controls. The controls were frequency matched with the cases regarding age (±5 years), sex and date and hospital of admission. The project was approved by the hospital Ethics Committee and written informed consent was obtained from all patients.

Methods

The cases and controls were interviewed at the hospital about their history of alcohol drinking using a standardized questionnaire, which showed high reproducibility when used in hospital settings in reliability studies (Corrao et al., 1994). Total alcohol intake was assessed, taking into account the period of maximum consumption before the onset of liver disease, and according to the average ethanol content of wine (12% in volume), beer (5%) and spirits (40%). An alcohol intake greater than 80 g/day was regarded as “heavy”. Patients were interviewed on history of blood transfusion in order to investigate the association between HCV genotypes and the route of transmission. We considered only transfusions received before 1980, i.e., 15 years or more before the first diagnosis of HCC, as a minimum interval between the possible onset of infection and HCC development.

A 10 ml blood sample was taken from all subjects and serum was stored at ~80°C until tested. The presence of HBV markers was assessed, taking into account the period of maximum consumption before the onset of liver disease, and according to the average ethanol content of wine (12% in volume), beer (5%) and spirits (40%). An alcohol intake greater than 80 g/day was regarded as “heavy”. Patients were interviewed on history of blood transfusion in order to investigate the association between HCV genotypes and the route of transmission. We considered only transfusions received before 1980, i.e., 15 years or more before the first diagnosis of HCC, as a minimum interval between the possible onset of infection and HCC development.

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Total RNA extracted from 100 μl of serum using the guanidium isothiocyanate-phenol-chloroform method was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR), using nested primers of the 5’ non-coding region. Genotypes of HCV were identified by amplification with nested PCR by means of type-specific primers of the core and NS5b regions (Donato et al., 1997) and classified according to the nomenclature of Simmonds et al. (1994).

Case and control sera positive for genotype 2 were also tested by means of subtype 2c-specific primers of the core region: 2c555 (sense: 5’-ACG GAC ATG ATG ATG AAC TGG-3’) and E 1818 (antisense: 5’-TAC CCA AGT TGC GGG ATC TAT 3’). They were derived from a database for HCV sequences accessible through the WWW at http://www.hiv.lanl.gov, including 48 full HCV genome sequences and 205 sequences coding for core region, in February 1998.

Assignment to subtype 2c of subjects infected with type 2 was checked by direct nucleotide sequence analysis of nested PCR products from the envelope 1 region obtained by the following oligonucleotide primers. The first-round PCR was performed with primer E 1278 (sense: 5’-RMT WCG GGH CAC CGC ATG G 3’) and E 1818 (antisense: 5’-GTC GAG GKG SGT AGT GCC AG 3’); the second-round PCR was performed with primer E 1300 (sense: 5’-GG GAC ATG ATG ATG AAC ACG TGG 3’) and E 1604 (antisense: 5’-GTG CCA RCT GCC RTT GGT G 3’).

Sequence analysis was restricted to 33 (25 cases and 8 controls) of 51 subjects infected with genotype 2 for whom serum samples were available, and 155 nt sequence between nucleotide position 5136 and 1490 at the 3’ end of HCV envelope 1 coding region was analyzed.

The odds ratios (ORs) and their 95% confidence intervals (95% CIs) were computed by unconditional logistic regression analysis using the maximum likelihood method. Sex, age and area of residence (town of Brescia vs. rest of province) were included in logistic regression models as possible confounders. The interaction between HCV and each of the other risk factors was evaluated by fitting both additive and multiplicative models. Since both models fitted the data adequately, we preferred to use the additive one as it allows practical and biological interpretation of the interactive effects of epidemiological research, according to Rothman (1986). Therefore, the interaction between HCV infection and each of the other factors was assessed by computing the synergy index (S) based on the additivity model. A value of S equal or similar to the unit was interpreted as indicative of additivity, whereas a higher value was taken as indicating that the effect of the joint exposures was greater than the sum of the separate effects.

### RESULTS

Table I shows the distribution of subjects by sex, age and residence. No difference was evident between cases and controls according to sex and age, whereas a slightly higher proportion of cases lived in towns in the province other than Brescia.

The distribution of subjects according to HCV and HBV markers and alcohol intake, and the corresponding ORs estimated by logistic regression models including age, sex and residence as possible confounders are shown in Table II. Anti-HCV positivity showed a strong association with HCC when HCV RNA was detected in serum but no association when it was not detectable (OR = 1.5, p > 0.1). Genotype 1b was the most common, followed by type 2, among HCC cases (68.0% and 29.5%, respectively, of the total HCV RNA+ cases), while types 1b and 2 were distributed equally among the controls. Nucleotide sequence analysis of HCV envelope 1 identified subtype 2c in all but 1 case and all the controls. One HCC case was of subtype 2a.

The OR for HCV genotype 1b (OR = 34.2) was more than 2-fold greater than that found for genotype 2. Of the HBV serological markers investigated, a strong association was found for HBsAg positivity, a weak, though significant, association for anti-HBc positivity alone and no association for anti-HBs positivity, with or without anti-HBc positivity. Alcohol intake was associated with HCC and a dose-response relationship was evident.

### Table I – Demographic Characteristics of Cases and Controls

| Demographic characteristics | Cases (n = 205) | Controls (n = 418) |
|-----------------------------|----------------|-------------------|
| Sex                         |                |                   |
| Male                        | 124 (60.5)     | 172 (41.5)        |
| Female                      | 71 (39.5)      | 246 (58.5)        |
| Age (years)                 |                |                   |
| 40–49                       | 12 (5.9)       | 12 (2.9)          |
| 50–59                       | 43 (21.0)      | 78 (18.6)         |
| 60–69                       | 93 (45.3)      | 140 (33.6)        |
| 70–75                       | 44 (21.4)      | 17 (4.1)          |
| Residence                   |                |                   |
| City of Brescia             | 140 (68.0)     | 140 (33.6)        |
| Rest of the province        | 65 (32.0)      | 278 (66.4)        |

### Table II – Distribution of Cases and Controls According to HCV and HBV Status and History of Alcohol Intake

| Risk factor               | Cases (%) | Controls (%) | OR (95% CI) |
|---------------------------|-----------|--------------|-------------|
| HCV status                |           |              |             |
| Anti-HCV−                 | 176 (57.7)| 567 (93.0)   | Reference   |
| Anti-HCV+ and HCV RNA−    | 7 (2.3)   | 13 (2.1)     | 1.5 (0.5–4.5)|
| Anti-HCV+ and HCV RNA+    | 122 (40.0)| 30 (4.9)     | 26.3 (15.8–44.0)|
| HCV genotypes             |           |              |             |
| 1a                         | 3 (1.0)   | 0 (0.0)      |             |
| 1b                         | 83 (27.2) | 15 (2.5)     | 34.2 (18.0–64.7)|
| 2                          | 36 (11.8) | 15 (2.5)     | 14.4 (7.2–28.7)|
| HBV status                |           |              |             |
| HBsAg−, anti-HBc−, anti-HBs− | 75 (24.8)   | 229 (37.9) | Reference   |
| Anti-HBs−, anti-HBc        | 89 (29.4) | 231 (38.2)  | 1.2 (0.8–1.9)|
| Anti-HBs− alone            | 3 (1.0)   | 40 (6.6)     | 0.3 (0.1–1.1)|
| Anti-HBc− alone            | 62 (20.5) | 79 (13.1)    | 1.9 (1.1–3.2)|
| HBsAg+                     | 74 (24.4) | 25 (4.1)     | 18.8 (10.0–35.2)|
| Alcohol intake (g/day)     |           |              |             |
| 0–40                       | 78 (26.1) | 50 (12.1)    | 3.3 (0.9–11.0)|
| 41–80                      | 59 (19.7) | 164 (39.4)   | 1.7 (0.9–2.9)|
| >80                        | 162 (54.2)| 208 (48.5)   | 7.1 (4.1–12.2)|

1 OR estimates (95% CI) adjusted for sex, age, residence and the risk factors in the table by unconditional logistic regression.
with a small non-significant OR increase for 41–80 g/day and a significant OR increase for higher values.

Analysis of HCC cases showed that patients with 1b infection did not differ from those with genotype 2 infection according to sex, age, residence, HBsAg positivity and intake of >80 g/day of ethanol ($p > 0.1$ for each comparison) (Table III).

A total of 26.3% of HCC cases and 15.6% of controls had received at least 1 blood unit during their lifetime. Considering only transfusion received by the HCC cases before 1980, a positive transfusion history was found among 23.6% of the patients with genotype 1b and 20.8% of those with genotype 2 ($p > 0.1$) (Table III).

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The distribution of HCC cases and controls and their mean ages at diagnosis (cases) or recruitment (controls) were analyzed according to HBsAg and HCV RNA and alcohol intake ($>80$ g/day). Only 32 HCC cases (10.5%) but 360 controls (59%) were negative for each factor. The mean age at diagnosis in HCC cases was lower among patients with HBsAg and heavy alcohol intake (59.7 years), with HBsAg and HCV RNA (61.4 years) and with HBsAg and HCV RNA and heavy alcohol intake (59.5 years), with respect to all of the other cases.

The relations between HCV infection and each of the other risk factors are shown in Table IV. Based on the additive model, HCV RNA positivity showed a synergism with HBsAg ($S = 2.4$) and with alcohol intake, especially for intake $>80$ g/day ($S = 4.0$).

The effect of alcohol drinking on the OR for HCV RNA positivity was also evaluated after excluding HBsAg$^+$ subjects. A dose-effect relationship between alcohol intake and the OR for HCC was evident among both HCV RNA$^+$ and HCV RNA$^-$ subjects (Fig. 1). HCV RNA positivity was associated with a significant increase in the OR for each alcohol intake category. The dose-response curves of the ORs for alcohol intake in HCV$^+$ and HCV$^-$ subjects were parallel on a logarithmic scale. A similar increase in the OR for alcohol intake was found when moving from the baseline (null-low) to the intermediate, and from the intermediate to the high category of consumption, in patients with and without HCV infection.

**DISCUSSION**

The main results of our study are: 1) subjects seropositive for anti-HCV but negative for HCV RNA are not at an increased risk of having HCC than those who are anti-HCV negative; 2) the risk of HCC by HCV infection is higher for genotype 1b with respect to type 2; 3) the risk for HCV infection increases substantially when other risk factors for HCC, such as HBsAg positivity and alcohol drinking, are present.

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**TABLE III** – DISTRIBUTION OF HCV GENOTYPES AMONG HCC CASES ACCORDING TO SEX, AGE, RESIDENCE, HBV STATUS AND HISTORY OF INTAKE OF 80 G/DAY OF ETHANOL

|   | la | lb | 2  |
|---|----|----|----|
|   | Number (%) | Number (%) | Number (%) |
| Total | 3 (100) | 83 (100) | 36 (100) |
| Sex | | | |
| Male | 2 (66.7) | 62 (74.7) | 25 (69.4) |
| Female | 1 (33.3) | 21 (25.3) | 11 (30.6) |
| Age (years) | | | |
| 40–49 | 1 (33.3) | 4 (4.8) | 0 — |
| 50–59 | 1 (33.3) | 18 (21.7) | 6 (16.7) |
| 60–69 | 1 (33.3) | 32 (38.6) | 19 (52.8) |
| 70–75 | 0 — | 29 (34.9) | 11 (30.6) |
| Mean (SD) | 55.0 (11.5) | 64.0 (8.3) | 66.5 (5.6) |
| Residence | | | |
| Town of Brescia | 3 (100) | 28 (33.7) | 16 (44.4) |
| Rest of the province | 0 — | 55 (66.3) | 20 (55.6) |
| HBsAg | | | |
| — | 2 (66.7) | 77 (92.8) | 32 (88.9) |
| + | 1 (33.3) | 6 (7.2) | 4 (11.1) |
| Intake of >80 g/day of ethanol | | | |
| No | 2 (66.7) | 52 (62.7) | 26 (72.2) |
| Yes | 1 (33.3) | 31 (37.3) | 10 (27.8) |

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**TABLE IV** – INTERACTION BETWEEN HCV INFECTION AND HBV STATUS AND ALCOHOL INTAKE

| Risk factors | Anti-HCV$^-$ and anti-HCV$^+$, HCV RNA$^-$ | HCV RNA$^+$ | Synergy index ($S$) |
|--------------|------------------------------------------|-------------|---------------------|
| HBV status   | Reference | 36/8 (14.5–87.1) | 35.6 (14.5–87.1) |
| HBsAg$^-$, anti-HBc$^-$, anti-HBs$^-$ | 47/221 | 1.3 (0.8–2.1) | 36.5 (16.0–83.1) | 1.0 |
| Anti-HBs$^+$, anti-HBc$^-$ | 42/10 | 2/1 | 36.5 (16.0–83.1) | 1.0 |
| Anti-HBs$^+$ alone | 32/69 | 1.7 (1.0–2.9) | 29.8 (10.9–56.8) | 0.8 |
| Anti-HBc$^-$ alone | 32/69 | 1.7 (1.0–2.9) | 29.8 (10.9–56.8) | 0.8 |
| HBsAg$^+$ | 63/24 | 21.1 (11.1–40.0) | 11/1 | 132 (15.3–890) | 2.4 |
| Alcohol intake (g/day) | | | | |
| 0–40 | 31/219 | Reference | 47/18 | 26.1 (12.6–54.0) |
| 41–80 | 27/157 | 1.5 (0.7–2.9) | 32/7 | 62.6 (23.3–168) |
| >80 | 120/203 | 7.3 (4.0–13.1) | 42/5 | 126 (42.8–373) | 4.0 |

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1OR estimates (95% CI) adjusted for sex, age and residence by unconditional logistic regression.
Concerning the first point, our results show that anti-HCV+ subjects with no detectable HCV RNA in serum do not have an increased risk of HCC. These findings are consistent with previous studies showing that anti-HCV+ subjects with persistently normal liver tests and no detectable HCV RNA in serum have recovered from HCV infection (Shakil et al., 1995).

Second, the different ORs found for HCV genotypes 1b and 2 confirm our data concerning the first 172 HCC cases and 332 controls enrolled in this study (Donato et al., 1997). The results of various studies performed on this issue are conflicting. Some of the discrepancies may depend on the study design. On one hand, case-control studies including subjects without clinically evident liver disease as controls have shown a 2- to 3-fold higher relative risk of HCC for 1b than for 2 genotype (Tanaka et al., 1996; Hatzakis et al., 1996; present study). Similarly, a higher prevalence of abnormal ALT levels has been found among subjects infected with HCV genotype 1b than in those infected with type 2 (Tagger et al., 1997). On the other hand, most cross-sectional studies found no difference in genotype prevalence between patients with chronic hepatitis, cirrhosis and HCC, after adjusting for age. Prospective studies among patients with chronic hepatitis or cirrhosis have shown contrasting results (Kobayashi et al., 1996; Bruno et al., 1997).

The apparent association between HCV genotype 1b and advanced liver disease may depend on a longer duration of infection. However, we did not find any differences in mean age between subjects with 1b and 2 genotype infection, in agreement with a survey carried out in the general Italian population (Guadagnino et al., 1997). Selection bias, confounding factors and inclusion criteria have also been suggested to explain the discrepancies (Bréchot, 1997). In our study, the HCC cases and controls were representative of total HCC cases occurring in the province of Brescia and of the general population in the same area, respectively. In fact, prevalences of HBsAg and anti-HCV positivities among HCC cases were similar with those found in another case-control study (Stroffolini et al., 1992) and prevalences among controls were similar with those observed in the general Italian population of the same age and sex (Guadagnino et al., 1997).

The marked heterogeneity of genotype 2 has been shown to include more than one subtype (Bréchot, 1997). However, we have demonstrated that all but 1 HCC case and all the controls were identified as infected with genotype 2, and 1 HCC case was infected with subtype 2a. This is in agreement with other studies showing that subtype 2c accounts for more than 90% of type 2 infections in Italy (Guadagnino et al., 1997; Maggi et al., 1997).

Another hypothesis is that HCV genotypes may affect the occurrence of chronic infection at a different rate, as suggested by a recent prospective study showing a higher chronicity rate for genotype 1b vs. the other types (Amoroso et al., 1998). If this is true, HCV genotypes might affect the risk of having HCC acting mainly in the early stages of carcinogenesis, by determining the occurrence of chronic hepatitis, while subsequent evolution toward cirrhosis and HCC would be independent of the genotype.

A third finding of our study is the synergism shown between HCV infection and positivity for HBsAg and alcohol drinking, according to an additive model of carcinogenesis. Synergy between HBV and HCV infections has been demonstrated (Donato et al., 1998), but it is very rare due to interference between the 2 viruses: in our study, 11 HCC cases (3.6%) and only 1 control (0.2%) showed both HCV RNA and HBsAg seropositivities. We also found a slight increase in the OR by anti-HBc positivity alone, probably because at least some of the subjects with such serological profile harbor the virus in the liver. However, no interaction between HCV infection and anti-HBc positivity alone was observed ($S = 0.8$; Table IV), in agreement with a recent cohort study among patients with HCV chronic hepatitis or cirrhosis showing a similar proportion of subjects developing HCC, among those untreated with interferon, in patients with or without anti-HBc positivity (International Interferon-α Hepatocellular Carcinoma Study Group, 1998). These findings suggest that when the 2 viruses replicate actively (both HBsAg and HCV RNA detectable in serum) they can cause more severe liver damage than by each infection alone. On the contrary, HBV does not appear to contribute substantially to the progression of the disease in subjects positive for HCV RNA with anti-HBs or anti-HBc alone in their serum.

The synergism between HCV and alcohol drinking appears to be by far the most relevant in terms of public health in countries where both HCV infection and alcohol intake are widespread, as is the Mediterranean area. Alcohol is a carcinogen for various human organs, including the liver. However, drinking a low amount of ethanol may be safe, since it has not been found to significantly increase the risk of liver disease, whereas it appears to reduce the overall risk of death. However, many figures suggest that if HCV infection and alcohol intake are both present, they can increase each other’s capacity to cause liver damage. Studies conducted among subjects with HCV liver diseases have shown that those with cirrhosis or HCC had a greater alcohol intake than those with less advanced disease (Roudot-Thoraval et al., 1997), and that alcohol intake increases the rate of fibrosis progression (Poynard et al., 1997) and the rate of carcinogenesis (Keda et al., 1998). Reciprocally, studies among alcoholics showed a higher severity of liver disease and a higher risk of developing HCC in the presence of HCV infection (Mendenhall et al., 1991). However, these studies used different cutoff levels of alcohol consumption and did not show a dose-effect relationship of liver disease and alcohol intake, according to the presence of HCV infection.

Our results show that alcohol drinking had a “pure” effect in increasing the risk of having HCC, independently of other disease determinants, and that the presence of HCV infection modified the risk due to alcohol intake. Reciprocally, alcohol intake increased the risk due to HCV infection by about 2-fold for alcohol consumption of 41–80 g/day and about 4-fold for consumption >80 g/day. These results are in line with those of Corrao and Aricò (1998) which show a synergism between HCV infection and alcohol intake >50 g/day on the risk of symptomatic liver cirrhosis. On the whole, these findings suggest that an alcohol intake of 41–80 g/day cannot be considered safe for people with HCV infection and that subjects with HCV chronic infection should abstain from alcohol consumption.

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