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Hepatocellular carcinoma in chronic HBV-HCV co-infection is correlated to fibrosis and disease duration

Rosa Zampino,* Maria A. Pisaturo,† Grazia Cirillo,‡ Aldo Marrone,* Margherita Macera,§ Luca Rinaldi,* Maria Stanzione,§ Emanuele Durante-Mangoni,|| Ivan Gentile,¶ Evangelista Sagnelli,† Giuseppe Signoriello,† Emanuele Miraglia del Giudice,‡ Luigi E. Adinolfi,* Nicola Coppola†

* Internal Medicine and Hepatology, † Department of Mental Health and Public Medicine, Section of Infectious Diseases, ‡ Department of Pediatrics, § Department of Clinical and Experimental Medicine and Surgery F. Magrassi e A. Lanzara, || Internal Medicine Monaldi Hospital, ¶ Second University of Naples; Department of Clinical Medicine and Surgery-University of Naples Federico.

ABSTRACT

Hepatocellular carcinoma (HCC) is a development of severe liver disease frequently due to HBV and/or HCV infection. The aim of this retrospective study was to evaluate the development of HCC in patients with HBV-HCV chronic infection compared with patients with single HBV or HCV infection and the viral and host factors correlated to HCC in co-infected patients. We studied 268 patients with histology proven chronic hepatitis: 56 had HBV-HCV co-infection (HBV-HCV group), 46 had HBV infection (HBV group) and 166 had HCV infection (HCV group). Patients were followed up for at least 3 years. Viral and host factors were studied. HCC was more frequent in HBV-HCV group (14%) compared with HBV (2%, p = 0.006) and HCV mono-infected (4%, p = 0.006). The Mantel-Haenszel test used to investigate the relationship between HBV-HCV co-infection and development of HCC indicated an association between development of HCC and HBV-HCV co-infection (p < 0.001). In the HBV-HCV group, patients with HCC were significantly older (p = 0.000), had longer disease duration (p = 0.001), higher blood glucose levels (p = 0.001), lower levels of steatosis (p = 0.02), higher levels of fibrosis (p = 0.000), higher HCV RNA (p = 0.01) than those without HCC. ALT, lipid profile, PNPLA3 variant distribution and HBV viral load did not differ among co-infected patients with or without HCC. In conclusion HCC was more frequent in our patients with HBV-HCV co-infection, than in those with HBV or HCV mono-infection; possible associated risk factors for HCC development seem a long duration of disease, high levels of fibrosis and carbohydrate intolerance.

Key words. HCC. Risk factors. Chronic hepatitis.

INTRODUCTION

Hepatocellular carcinoma is a common cancer with a medium incidence in Italy (10.0 to 20.0 cases per 100,000 individuals).[1]

Liver cirrhosis is present in about 80-90% of patients with HCC, and viral infection by hepatitis B and/or C is frequently the cause of liver disease progression and evolution to HCC.[2]

In HBV infection most cases of HCC are observed in cirrhotic patients,[3,4] especially when a high viral load is present,[5] but HBV can cause HCC in the absence of cirrhosis. The HBV genome variants have shown different behavior in different geographical areas, but it seems almost certain that HBV genotype B is associated with HCC in young people without cirrhosis.[6]

In HCV-infected patients, the frequency of HCC ranges from 1 to 3% over 30 years, rising to an annual rate of 1-8% when cirrhosis is present.[1] HCV viremia is suggested as a risk factor for HCC.[7] Also liver steatosis, a characteristic feature of chronic hepatitis C,[8] has been identified as an independent risk factor for the development of HCC in HCV-infected patients.[9]

Recently, a polymorphism of the patatin-like phospholipase domain-containing 3 (PNPLA3) gene, involved in the lipid metabolism, has been associated with liver steatosis in NAFLD,[10] chronic hepatitis B,[11] chronic hepatitis C[12,13] and also with HCC.[12,14]

Correspondence and reprint request: Rosa Zampino, M.D. Ph.D.
Internal Medicine and Hepatology
Second University of Naples
Via Pansini, 5 - Edificio 10, 80131 Naples, Italy
Tel.: 0039 (081) 5666225. Fax 0039 (081) 5666230.
E-mail: rosa.zampino@unina2.it

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HBV-HCV chronic co-infection correlated with a more severe liver disease[15-18] and a more rapid progression to liver cirrhosis and HCC.[19-22] However, a meta-analysis of more recent studies revealed a lower oncogenic effect in co-infected patients,[23] perhaps related to the mutual interference between HBV and HCV.

The aim of the present retrospective study was to evaluate the development of HCC in a group of patients from Southern Italy with chronic HBV-HCV co-infection compared with patients with a single HBV or HCV infection and to identify possible viral (viral load, viral genotypes) and host (anthropometry, biochemistry, histology, PNPLA3 polymorphisms, clinical history) parameters correlated to HCC in co-infected patients.

MATERIAL AND METHODS

This is a retrospective study involving five Liver Units in Naples, Southern Italy, which have cooperated in several clinical investigations using the same clinical approach and the same laboratory methods.

Included in the study were patients with presence of HBsAg and/or anti-HCV in serum for at least one year at the time they underwent their first liver biopsy proving chronic hepatitis and had a documented clinical, serological and virological follow-up of at least 3 years after liver biopsy. Patients positive for anti-HIV and/or anti-HDV and with other causes of liver disease were excluded.

Because we are not able to define exactly the moment of infection for all patients, the presumed time of acquisition of the disease was considered as the first time ALT elevation was recorded in the medical history of all patients. Furthermore, because the interval between the first ALT elevation and liver biopsy varied, we considered the baseline as the time of the first liver biopsy.

During their disease history patients underwent liver biopsy, if clinically indicated. Liver specimens were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin and Masson's trichrome stain. Liver biopsies were examined by a pathologist who, unaware of the clinical and laboratory data, used Ishak's scoring system to grade necroinflammation and fibrosis[24] and a homemade scoring system for steatosis he had been using for decades (score 1 = 1-10% of hepatocytes with fatty deposition, score 2 = 11-30%; score 3 = 31-60%; score 4 ≥ 60%).[8]

In accordance with the routine admission protocol established over the years by the liver units involved in the present study, at the time of the liver biopsy all patients underwent physical examination, full liver function tests, blood cell counts, HDV, HIV serum markers and liver ultrasound scan. A questionnaire on alcohol and drug consumption was submitted to patients for completion. Alcohol abuse was defined as > 30 g/die no later than 6 months before entering the study. For all patients body mass index (BMI: kg/m^2), waist circumference, blood fasting glucose, triglycerides and cholesterol were also determined.

Samples of serum, plasma and whole blood were obtained for each patient on the day of the liver biopsy and stored at -80 °C. The polymorphisms of the PNPLA3 gene were tested on these stored blood samples.

All patients enrolled were followed up for at least 3 years (range 3-51) from the actual or estimated time of infection, with evaluation of liver function tests, abdominal ultrasound and alpha-fetoprotein and serological (HBsAg, anti-HCV) and virological (HBV DNA and HCV RNA) tests.

All procedures used in the study were in accordance with the international guidelines, with the standards on human experimentation of the Ethics Committee of the Second University of Naples and with the Helsinki Declaration of 1975 and revised in 1983. The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria of the Second University of Naples. All patients signed an informed consent for the collection and storage of plasma samples and for the collection and use of their data in clinical research.

- **Serological determinations.** Serum markers for HBV, HCV, HDV and HIV infection were sought in serum using commercially available immunoenzymatic assays (Abbott Laboratories, North Chicago, IL and Ortho Diagnostic Systems, Raritan, NJ).
- **HBV and HCV genotype and viral load.** HBV genotypes were determined by phylogenetic analysis of sequences of 400 nt of the S region, as previously described.[29] HCV genotypes were determined using the VERSANT HCV genotype 2 LIPA (Siemens, Erlangen, Germany), following the manufacturer's instructions.

HBV DNA and HCV RNA were sought in plasma of all patients in the study, as previously described.[29,30] The detection limit of HBV DNA is estimated at around 40 copies/mL and that of HCV RNA at around 40 IU/mL.

PNPLA3 polymorphisms

Genomic DNA was extracted from whole blood by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and analyzed for PNPLA3 polymorphisms.

All patients were genotyped for the PNPLA3 rs738409 C to G variant underlying the I148M substitution. The following primers were used, F: 5'-GCCCTGCTCACTTGGAGAAA-3' and R: 5'-TGAAAGGCAGTGAGGCATGG-3'. The FokI restriction enzyme, as previously described, was used to identify the variant, since the G allele eliminates a FokI restriction site. Random samples were confirmed by direct genotyping, which provided concordant results in all cases.[31]

- **Statistical analysis.** Comparison between groups was made applying the Mann-Whitney U test for continuous variables and the t-test or χ^2 test for categorical data. A P value of < 0.05 was considered significant. The Mantel-Haenszel test was used to estimate the common odds ratio and to test the association of HCC and the presence of HBV-HCV co-infection in relation to the disease duration. The statistical analyses were performed using SPSS statistical software v. 17.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Two hundred and sixty-eight Caucasian patients with histology proven chronic hepatitis entered the study: 56 had HBV-HCV co-infection (HBsAg/HBVDNA/anti-HCV/HCVRNA-positive; HBV-HCV group), 46 had HBV infection (HBsAg/HBVDNA-positive; HBV group) and 166 had HCV infection (anti-HCV/HCVRNA-positive; HCV group).

Table 1 shows the general characteristics of the HBV-HCV group compared with the mono-infected groups. Patients were homogeneous for age, BMI, waist circumference, cholesterol and were prevalently males in the 3 groups.

Past alcohol abuse and injection drug use were indicated as they are factors that potentially contribute to liver injury and carcinogenesis; alcohol abuse was significantly higher in the HCV group ($p = 0.029$) (Table 1).

ALT and AST were significantly lower in the co-infected than in the HBV group ($p = 0.00$ and $p = 0.02$, respectively) (Table 1). ALT and fasting glucose were significantly lower in the co-infected than in the HCV mono-infected group ($p = 0.00$ and $p = 0.056$, respectively). Triglycerides were significantly higher in the co-infected than in the HBV monoinfected ($p = 0.009$).

In the HBV-HCV group 8/56 (14%) patients were HBeAg-positive, while in the HBV group no patient was HBeAg-positive. In the HBV-HCV co-infected group, HBV DNA and HCV RNA were significantly lower than in the HBV and HCV mono-infected groups, respectively ($p = 0.0001$, $p = 0.000$). No difference in the distribution of HCV and HBV genotypes was observed between the HBV-HCV group and the HCV and HBV groups.

In addition, the HBV-HCV group presented lower steatosis ($p = 0.053$) than the HCV group, but significantly higher fibrosis ($p = 0.03$) (Table 1). Table 2 shows the specific distribution of HAI, fibrosis and steatosis for the three groups. A higher number of HBV-HCV co-infected patients presented severe fibrosis levels ($p = 0.002$) and less severe steatosis score ($p = 0.03$) compared to the HCV mono-infected.

PNPLA3 polymorphisms CC and GG were more frequently present in patients with HCV infection (Table 1).

During the clinical follow-up, 199 (74%) of the 268 patients received antiviral treatment, established by the physicians in care on the basis of the plasma expression of HBV and HCV replication, liver histology and current treatment guidelines.[25-27] The diagnosis of HCC was made according to the AASLD management guidelines.[28]

HCC development

HCC was more frequent in the HBV-HCV group (14%) compared to the HBV (2%, $p = 0.006$) and HCV mono-infected groups (4%, $p = 0.006$). On the basis of the previously reported data on the emergence of HCC in each single infection,[1,7] we evaluated the emergence of HCC in relation to the duration of disease $<$ or ≥ 25 years. Excluding the confounding effect of disease duration, the Mantel-Haenszel test was used (Table 3) to investigate the relationship between HBV-HCV chronic co-infection and the development of HCC. Of the 139 patients with a disease duration less than 25 years, the development of HCC was observed in 1 of the 27 in the HBV-HCV group, in 1 of the 27 in the HBV group and in 2 of the 85 in the HCV group; of the 129 patients with a disease duration of 25 years or more, the development of HCC was observed in 7 of the 29 in the HBV-HCV group, in none of the 19 in the HBV group and in 4 of the 81 in the HCV group (Table 3), indicating an association between HBV-HCV co-infection and the development of HCC ($p < 0.008$).

Table 1. General characteristics of the HBV-HCV group *versus* the single HBV and HCV groups at time of first liver biopsy

	HBV-HCV	HBV	P HBV-HCV *vs.* HBV	HCV	P HBV-HCV *vs.* HCV
Patients, n°	56	46		166	
Median age (range)	49 (25-76)	47 (23-65)	0.7	53.5 (21-80)	0.4
Males, n° (%)	34 (60)	33 (70)	0.3	95 (57)	0.7
with disease duration >30 yrs, n° (%)	10(18)	7 (15)	0.9	59 (35)	0.02
Alcohol abusers (> 30g/die), n° (%)	3 (5)	1 (2)	0.09	31 (19)	0.029
Injection drug users, n° (%)	5 (9)	1 (2)	0.3	24 (14)	0.4
BMI (mean ± SD)	25.7 ± 3	26 ± 4.5	0.68	26 ± 3.8	0.59
Waist circumference (mean ± SD)	91.7 ± 9.8	95.4 ± 12.8	0.1	91.4 ± 11	0.8
Glucose (mean ± SD) mg/dL	90 ± 13.4	85.8 ± 14.4	0.1	96 ± 22	0.056
AST (mean ± SD) IU/L	55 ± 39	83 ± 84	0.02	62 ± 50	0.03
ALT (mean ± SD), IU/L	28 ± 62.5	124.95 ± 92	0.00	89 ± 73	0.00
Cholesterol (mean ± SD) mg/dL	182 ± 34	182 ± 31	1	182 ± 41	1
Triglycerides (mean ± SD) mg/dL	109 ± 55	85 ± 29	0.009	103 ± 53	0.4
Median HCV RNA (range) IU/mL	1.15 x 10^5 (120- 6.4 x 10^5)	-		8 x 10^5 (2,818-7 x 10^7)	0.000
HCV genotype 1, n° (%)	41 (74)			112 (68)	0.5
HCV genotype 2, n° (%)	7 (12)			37 (22)	0.4
HCV genotype 3, n° (%)	8 (14)			17 (10)	0.5
HBV genotype A, n° (%)	1 (2)	7 (4)	0.6		
HBV genotype D, n° (%)	55 (98)	159 (96)			
Median HBV DNA (range) IU/mL	1.9 x 10^3 (1,500-10 x 10^7)	2 x 10^5 (3,000-1 x 10^8)	0.0001		
HAI (mean ± SD)	5.5 ± 2.8	6.1 ± 3.4	0.3	6 ± 3.6	0.3
Fibrosis (mean ± SD)	2.8 ± 1.8	2.9 ± 1.3	0.7	2.3 ± 1.4	0.03
Steatosis (mean ± SD)	0.9 ± 0.85	1.2 ± 1	0.1	1.25 ± 1.25	0.053
PNPLA3 - CC, n° (%)	33 (59)	21 (46)	0.2	65 (39)	0.01
PNPLA3 - GC, n° (%)	21 (37.5)	20 (44)	0.6	81 (49)	0.2
PNPLA3 - GG, n° (%)	1 (1.8)	5 (10)	0.1	20 (12)	0.04
HCC n° (%)	8 (14)	1 (2)	0.006	6 (4)	0.006

HAI: Histological activity index.

Table 2. Histology distribution in the three groups of patients.

	HBV-HCV n (%)	HBV n (%)	P HBV-HCV *vs.* HBV	HCV N (%)	P HBV-HCV *vs.* HCV
HAI					
Minimal	13 (23)	9 (20)	0.8	42 (26)	0.8
Mild	33 (59)	25 (54)	0.7	82 (49)	0.4
Moderate	10 (18)	7 (15)	0.9	33 (20)	0.8
Severe	0	5 (11)	0.039	9 (5)	0.1
Fibrosis					
Mild	30 (54)	18 (39)	0.2	96 (58)	0.6
Moderate	13 (23)	24 (52)	0.05	58 (35)	0.1
Severe (cirrhosis)	13 (23)	4 (9)	0.09	12 (7)	0.002
Steatosis					
Absent	19 (34)	11 (24)	0.3	53 (32)	0.9
Minimal	23 (41)	16 (35)	0.6	59 (36)	0.5
Mild	12 (22)	11 (24)	0.9	27 (16)	0.5
Moderate	2 (3)	7 (15)	0.08	11 (6)	0.6
Severe	0	1 (2)	0.9	16 (10)	0.03

HAI: Histological Activity Index.

Table 3. HCC development in relation to disease duration in the three groups of patients.

	Disease duration <25 years		Disease duration ≥ 25 years	
	HCC	No HCC	HCC	No HCC
HBV-HCV group	1/27 (3.7%)	26/27 (96.3%)	7/29 (24.1%)	22/29 (75.9%)
HBV group	1/27 (3.7%)	26/27 (96.3%)	0/19	19/19 (100%)
HCV group	2/85 (2.3%)	83/85 (97.7%)	4/81 (4.9%)	77/81 (95.1%)

P: 0.008.

Table 4. General characteristics of the HBV-HCV co-infected patients with and without HCC.

	HBV-HCV without HCC	HBV-HCV with HCC	P
Patients, n°	48	8	
Median age (range)	43 (25-76)	68 (59-76)	0.000
Males, n° (%)	27 (56%)	7 (88%)	0.1
Median disease duration yrs, (range)	21 (3-38)	34 (21-51)	0.001
Alcohol abusers (> 30 g/die), n° (%)	3 (6.25%)	0	0.9
Injection drug users, n° (%)	3 (6.25%)	1 (12%)	0.9
BMI (mean ± SD)	25.6 ± 3	26.5 ± 9	0.58
Waist circumference (mean ± SD)	91. ± 10	96.7 ± 5.4	0.1
Glucose (mean ± SD) mg/dL	87 ± 11	103 ± 19	0.001
AST (mean ± SD) U/L	39 ± 22	60 ± 44	0.03
ALT (mean ± SD), U/L	30 ± 65	58 ± 36	0.2
Cholesterol (mean ± SD) mg/dL	184 ± 35	171 ± 31	0.3
Triglycerides (mean ± SD) mg/dL	111 ± 59	100 ± 12	0.6
Median HCV RNA (range) IU/mL	2,900 (120-3.9 x 10^5)	3,857 (270- 2.7 x 10^3)	0.01
Median HBV DNA (range) IU/mL	2400 (1,500-1.70 x 10^7)	2,028 (1,530-1.3 x 10^3)	0.1
HAI (mean ± SD)	5.5 ± 2.9	5.7 ± 2.7	0.8
Fibrosis (mean ± SD)	2.4 ± 1.6	4.6 ± 1.9	0.000
Steatosis (mean ± SD)	0.9 ± 0.8	0.2 ± 0.5	0.02
PNPLA3 - CC, n° (%)	29 (60.5)	5 (62.5)	0.7
PNPLA3 - GC, n° (%)	18 (37.5)	3 (37.5)	0.7
PNPLA3 - GG, n° (%)	1 (2)	0	0.3
Patients with antiviral treatment	30 (62.5%)	7 (88%)	0.3

HAI: Histological activity index.

Analysis of baseline factors associated with the development of HCC in the HBV-HCV group

Table 4 shows the analysis of the baseline factors associated with the development of HCC in the HBV-HCV group. ALT, the lipid profile, PNPLA3 variant distribution and HBV viral load did not differ between patients with HCC and those without. Patients with HCC were significantly older (p = 0.000) than those without HCC, presented a longer disease duration (p = 0.001) and higher levels of HCVRNA (p = 0.01). They also showed higher blood glucose levels (p = 0.001); one (1%) patient in the HBV-HCV group, 2 (4%) in the HBV group and 13 (8%) in the HCV group had diabetes mellitus. The histological evaluation showed significantly lower levels of steatosis (p = 0.02) and higher levels of fibrosis (p = 0.000) in patients with HCC compared to those without. A significantly higher number of HBV-HCV co-infected patients with HCC presented severe fibrosis levels (p = 0.012) and an absence of steatosis (p = 0.04) compared to HBV-HCV co-infected patients without HCC (Table 5).

During their clinical history 62% of the patients without HCC and 88% of those with HCC had received treatment in accordance with the then current guidelines.

DISCUSSION

In the present study HCC was more frequent in HBV-HCV co-infected patients than in the mono-infected and, apparently, the longer the duration of the disease, the more severe fibrosis and the greater the development of HCC.

Table 5. Histology distribution in HBV-HCV infected patients with or without HCC

	HBV-HCV without HCC n (%)	HBV-HCV with HCC n (%)	p
HAI			
Minimal	13 (27)	1 (12)	0.6
Mild	26 (54)	5 (63)	0.9
Moderate	9 (19)	2 (25)	0.9
Severe	0	0	
Fibrosis			
Mild	30 (62)	1 (12)	0.017
Moderate	11 (22)	2 (25)	1.0
Severe (cirrhosis)	8 (16)	5 (63)	0.012
Steatosis			
Absent	15 (32)	6 (75)	0.04
Minimal	20 (42)	2 (25)	0.4
Mild	11 (22)	0 0.3	
Moderate	2 (4)	0 1.0	
Severe	0	0	-

HAI: Histological activity index.

In agreement with previous studies,[14,16-21] HBV-HCV co-infection correlated with a more severe liver disease, which frequently entails a more rapid evolution to cirrhosis and HCC. Unfortunately, there are no literature data from a single study on a large population to allow a better assessment of the risk of HCC development in patients with HBV-HCV co-infection. The results available emerge from meta-analyses on international studies, most of them early studies that considered patients from different parts of the world with different prevalences of viral infections.[19,21] Furthermore, the role of carcinogenetic factors, other than viruses, have to be taken into account in people of very different cultures and habits,[22] and older age and a longer duration of liver disease are important factors in the development of HCC also in patients with HBV and HCV mono-infection, particularly in developed countries.[32]

In our HBV-HCV co-infected patients with HCC, lower steatosis and higher fibrosis levels than in patients without HCC were observed. Previous studies have implicated steatosis as a risk factor for HCC, directly in HCV[8,33-34] and indirectly in HBV[35-37] infection. Molecular mechanisms to explain the role of liver steatosis in HBV or HCV carcinogenesis have been investigated; some hypotheses have been formulated to elucidate the role of HBV-HCV co-infection in the molecular pattern involved in lipid synthesis and metabolism, which can increase HCC development.[38] In contrast with these data, the histological picture of our patients showed low levels of steatosis. We may hypothesize that during the natural progression of chronic hepatitis, an evolution is possible in which steatosis is present in a first stage of the disease, followed by fibrosis[8] and eventually by HCC development.

The existence of a possible reciprocal interference between HBV and HCV that might control the development of liver steatosis is still unclear, but a predominant role of one virus in liver injury cannot be excluded.

During chronic viral hepatitis, factors such as alcohol abuse, treatment, etc. and genetic factors may influence the outcome of disease. Alcohol consumption is certainly the most important factor worsening chronic hepatitis progression. It is well known that alcohol is a risk factor for liver cancer[39] and that there is a synergistic effect of alcohol with HCV and HBV infection in promoting HCC.[40,41] In our population alcohol abuse was more frequent in patients with HCV infection than in the co-infected and no correlation with HCC development was observed in the HBV-HCV group. We enrolled patients who had no alcohol abuse for at least 6 months, but we can not quantify the effects of alcohol abuse in generating liver damage and predisposing to liver malignancy. Furthermore, the PNPLA3 variant showed no correlation with HCC.

Previous studies have demonstrated that viral suppression reduces, but does not eliminate, the risk of HCC both in HBV and HCV chronic infection.[42-45] We cannot assess the beneficial effects of antiviral

treatment in protecting our patients from the development of HCC, first because of the small number of patients, and secondly because they were treated at different times with different schedules as per the then current guidelines.

In our patients, fasting blood glucose was significantly higher in HBV-HCV co-infected patients with HCC than in those without. Previous studies have found an association between diabetes and HCC [46-48], more frequent in patients with HCV infection.[49,50] To our knowledge, no data are available for the HBV-HCV co-infected, but our results seem to confirm those of the literature for HCV patients. It is possible that HCV acts on the glucose metabolism, probably inducing insulin resistance also in the presence of HBV. However, this was not associated with elevated liver steatosis levels, which were not observed in our co-infected group, but it was accompanied by higher triglycerides levels.

CONCLUSION

In our patients, HCC was more frequent in the HBV-HCV co-infected than in those with HBV or HCV mono-infection; a long duration of disease, high levels of fibrosis and carbohydrate intolerance seem to be risk factors for the development of HCC. Further prospective investigations with a higher number of cases are needed to confirm these data.

DISCLOSURE

All authors have no conflict of interest in connection with this study.

AUTHOR CONTRIBUTIONS

Rosa Zampino and Nicola Coppola conceived and drafted the manuscript; Grazia Cirillo carried out the laboratory work, Maria Antonietta Pisaturo, Aldo Marrone, Margherita Macera, Luca Rinaldi, Maria Stanzione, Emanuele Durante-Mangoni and Ivan Gentile cooperated in the patients' enrolment and follow-up; Giuseppe Signoriello performed the statistical analysis; Evangelista Sagnelli, Emanuele Miraglia del Giudice and Luigi Elio Adinolfi critically reviewed the manuscript. All authors approved the final version of the manuscript.

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