After identifying HIV/HBV-coinfected patients, medical records including laboratory data of these patients were reviewed between the date of the oldest available record for these patients and the final date of the record acquired by the end of the study. The laboratory data at the diagnosis or first recognition of HBV infection and the latest data in the study period were compared for analysis unless otherwise noted. HBV genotypes (A through D) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV GENOTYPE EIA; Institute of Immunology, Tokyo, Japan) on the basis of the pattern of detection using monoclonal antibodies of a combination of epitopes on preS2-region products, each of which was specific for each genotype [22, 23].

Ethical issues

The respective ethics committees of the six hospitals approved the study. Informed consent was obtained from each study participant.

Statistical analyses

For the comparison of means of collected data, Student's *t* test (paired *t* test) was performed unless otherwise specified. The chi-square test was performed to determine the independence of clinical parameters.

Results

Two hundred and fifty-two patients were identified to have HIV/HBV coinfection. The mean age was 39.5 years, and the proportion of male patients was very high (243 of 252; 96.4 %). The main presumed transmission route of HIV was male homosexual contact (186 of 252; 73.8 %), followed by heterosexual contact. Among those HIV/HBV-coinfected patients, 21 of the 252 (8.3 %) acquired acute hepatitis during the study period (Table 1).

Table 1 Clinical background of HIV/HBV-coinfected patients

Number (male:female)	243:9
Age (year)	39.5 ± 9.6[a]
Presumed Transmission Route	
Transfusion	14
Homosexual contact	186
Heterosexual contact	24
Injection drug use	2
Others	4
Onset as acute hepatitis	21

[a] Mean ± standard deviation

The HBV genotype was determined in 77 patients. Among them, genotype A HBV was the most frequent (58 of 77; 75.3 %), followed far behind by genotype C (7 of 77; 9.1 %), which is the predominant genotype in the entire chronic hepatitis B population in Japan. Genotype B, which is also common in Japan, was found only in three patients (3.9 %). Genotype A was detected almost exclusively in homosexual patients (57 of 58; 98.3 %) (Fig. 1).

At the end of the study period, 113 patients (44.8 %) received some type of anti-HBV drug such as interferon, lamivudine, adefovir, or entecavir, not as part of anti-HIV treatment. Ninety-seven (38.5 %) patients were still taking anti-HBV drugs by the end of the study period. The median ALT level was 30.0 IU/l (5th percentile, 11.1; 95th percentile, 128.9), suggesting the existence of some liver injury. Liver function was normal in most HIV/HBV-coinfected patients. The mean serum albumin level was 4.1 ± 0.6 g/dl, and the median serum total bilirubin level was 0.8 mg/dl (5th percentile, 0.3; 95th percentile, 3.8). The mean platelet count was 21.0 ± 6.1 × 10^4/ml. The hepatitis B e antigen (HBeAg) was detected in 84 patients, and the HBV DNA level was high (higher than 100,000 IU/l) in 55 patients (Table 2). Three of the 252 (1.1 %) HIV/HBV-coinfected patients developed advanced chronic liver diseases, such as cirrhosis with the complication of ascites and/or hepatic encephalopathy, or hepatocellular carcinoma. Although we tried to retrieve information on alcohol consumption of the patients, it was available for only a limited number of patients (26 of 252); among the 26, only 2 patients had a habit of taking more than 60 g alcohol per day. The remaining 24 patients took alcohol only on social occasions. The antiretroviral agents used for these study patients are listed in detail in Table 3. Among those who had a known history of ART, 158 of 252 (62.7 %) received regimens that include anti-HBV drugs at least once previously, whereas 42 (16.7 %) did not, and no information is available for the remaining 52. The most common drug combination for HIV/HBV-coinfected patients was ATV/r + FTC/TDF (22 of 172; 12.8 %) (Table 4). FTC/TDF, composed of two drugs active against HBV, is recommended for HIV/HBV-coinfected patients

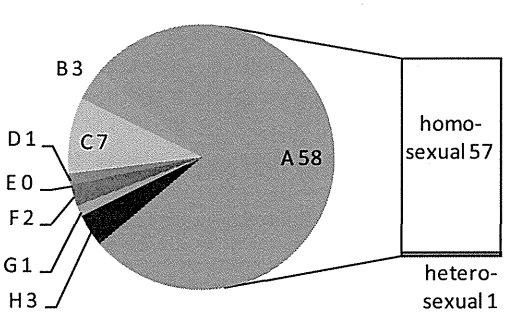

Fig. 1 Hepatitis B virus (HBV) genotype

Table 2 Liver function and related parameters of HIV/HBV-coinfected patients

Albumin (g/dl)	4.1 ± 0.6
Bilirubin[a] (mg/dl)	0.8 (5th percentile, 0.3; 95th percentile, 3.8)
ALT[a] (IU/l)	30.0 (5th percentile, 11.1; 95th percentile, 128.9)
WBC ($\times 10^3/\mu l$)	5.2 ± 1.6
Platelet ($\times 10^4/\mu l$)	21.0 ± 6.1
HBeAg (positive:negative)	84:68
HBV DNA (high:low)[b]	55:127

[a] Median and percentiles are provided instead of mean and standard deviation because of the nonnormality of the distribution

[b] HBV DNA level of 100,000 IU/l or higher is categorized as "high"

as one of the preferred NRTI backbones of the ART regimen [24].

We compared the clinical characteristics between patients who received the full ART and those who did not. Regarding the baseline statistical data, the observation period was longer for patients on ART, and there were more patients with AIDS in the ART group (10 of 64 vs. 52 of 162) (Table 5a). No significant difference was observed between the non-ART and ART groups in male/female ratio, age, transmission route, HBV markers, or advanced liver disease. Liver-related death was not observed, but hepatic failure with ascites and/or hepatic encephalopathy developed in 2 patients on ART and hepatocellular carcinoma developed in another patient.

Comparison between the ART group and the non-ART group revealed that the baseline liver function was worse in the ART group. At the beginning of the study period, the ART group showed a significantly lower CD4+ T-cell count than the non-ART group. The total white blood cell count and platelet count were also lower in the ART group. Although it is not statistically significant, the serum albumin level and prothrombin time (PT) index were lower in the ART group. However, at the end of the observation period, these parameters improved significantly in the ART group. The difference in CD4+ T-cell count between the ART and non-ART groups became marginal and became statistically insignificant (Table 5b).

Changes in the liver function of HIV/HBV-coinfected patients may not be fully explained by the changes in HBV activity because some parameters relevant to the estimation of liver function showed paradoxical changes. To clarify this observation, we compared the changes in liver function among HIV/HBV-coinfected patients on ART with respect to protease inhibitor (PI) use.

The mean serum total bilirubin level in patients on ART with PI use (PI group) at the beginning of the observation period was 1.1 mg/dl, whereas that in patients without PI use (non-PI group) was 0.8 mg/dl. The means at the end of the study period were 1.6 mg/dl in the PI group and 0.7 mg/dl in the non-PI group. Because the sample distribution of serum total bilirubin level did not follow the normal distribution by logarithmic transformation, we compared the means statistically. At the beginning, the difference in the mean between the PI group and the non-PI group was not significant ($p = 0.257$). At the end of the observation period, a statistically significant difference ($p = 0.001$) was observed. We then calculated the

Table 3 Antiretroviral treatment of HIV/HBV-coinfected patients

Antiretroviral drugs	Number of patients
NRTIs	
Zidovudine (AZT)	34
Didanosine (ddI)	9
Ddl / enteric coated	7
Zalcitabine (ddC)	1
Stavudine (d4T)	4
Lamivudine[a] (3TC)	84
Abacavir[3] (ABC)	38
Tenofovir[3] (TDF)	27
Emtricitabine (FTC) / TDF[a]	57
NNRTIs	
Nevirapine (NVP)	10
Efavirenz (EFV)	34
Delavirdine (DLV)	1
PIs	
Indinavir (IDV)	4
Ritonavir (RTV)	50
Nelfinavir (NFV)	8
Lopinavir (LPV)	3
Ritonavir-boosted LPV (LPV/r)	40
Atazanavir (ATV)	39
ATV/r	6
Fosamprenavir (FPV)	13

NRTI nucleoside reverse transcriptase inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor

[a] Agents with anti-HBV activity

Table 4 Antiretroviral regimens used for HIV/HBV-coinfected patients

Antiretroviral regimen	Number of patients
ATV/r + FTC/TDF	22
LPV/r + 3TC + TDF	8
LPV/r + FTC/TDF	7
EFV + FTC/TDF	6
ATV/r + 3TC + TDF	5

Table 5 Comparison of changes in clinical parameters of HIV/HBV-coinfected patients with or without antiretroviral therapy (ART)

a. Baseline statistical data	Natural course[a] (without ART)	With ART	*p* value (with vs. without ART)
Number (male:female)	84:6	159:3	0.105†
Age (year)	37.0 ± 10.3	39.0 ± 9.1	0.362
Observation period (month)	34.5 ± 55.5	50.9 ± 43.9	0.022*
Presumed transmission route	Blood products:homosexual contact:heterosexual contact:injection drug use:other		
	5:60:12:2:3	9:126:12:0:1	0.052†
Recognized acute hepatitis	10	11	0.243†
HBeAg (positive:negative)	42:18	100:40	0.394†
HBV DNA (high:low)	29:18	83:37	0.356†
HBV genotype	A:B:C:D:F:G:H		
	17:0:1:1:1:0:1	31:3:6:0:1:1:2	0.372†
Ascites	1/56	2/144	1.000†
Hepatocellular carcinoma	0/62	1/159	1.000†
Acquired immunodeficiency syndrome (AIDS)	10/64	52/162	0.012*,†

b. Comparison of clinical parameters between pre- and post-ART among patients with and without ART	Natural course (without ART)	With ART	*p* value (with vs. without ART)
CD4 count (per μl)			
Start[b]	402.9 ± 180.1	242.5 ± 187.6	0.000*
End[c]	406.4 ± 212.4	398.1 ± 195.9	0.883
p value (start vs. end)	0.893	0.000*	
Albumin (g/dl)			
Start	4.1 ± 0.4	3.8 ± 0.8	0.292
End	3.9 ± 0.8	4.2 ± 0.4	0.025*
p value	0.473	0.001*	
Bilirubin[d] (mg/dl)			
Start	0.7 (0.30, 4.26)	0.5 (0.30, 2.62)	0.138
End	0.5 (0.25, 1.30)	0.9 (0.36, 4.32)	0.000*
p value	0.046*	0.000*	
ALT[d] (IU/l)			
Start	46.0 (15.0, 1418.2)	34.0 (12.8, 1,068.8)	0.120
End	27.0 (9.9, 229.9)	31.5 (12.73, 89.3)	0.713
p value	0.003*	0.000*	
Prothrombin time index (%)			
Start	89.4 ± 13.1	78.8 ± 23.0	0.650
End	78.8 ± 27.3	84.2 ± 16.3	0.531
p value	0.377	0.218	
WBC ($\times 10^3/\mu l$)			
Start	6.1 ± 2.4	4.8 ± 2.1	0.000*
End	5.4 ± 1.4	5.1 ± 1.6	0.404
p value	0.044*	0.247	
Platelet ($\times 10^4$ /μl)			
Start	22.2 ± 6.5	19.3 ± 6.3	0.010*
End	21.2 ± 6.5	20.8 ± 6.1	0.649
p value	0.204	0.001*	

* $p < 0.05$

† Chi-square test was performed

[a] Two patients with habitual alcohol intake were included in this group

[b] Start of observation period

[c] End of observation period

[d] Means were compared by log transformation because of the nonnormality of the distribution; median and percentiles (5th percentile, 95th percentile) are provided

difference in serum total bilirubin level between the beginning and the end of the observation period [Dbilirubin level = (bilirubin level at the end) − (bilirubin level at the beginning)] in individual patients and compared it between the PI group and the non-PI group. The mean Dbilirubin level in the PI group was 0.5 ± 3.4 mg/dl and that in the non-PI group was −0.2 ± 1.6 mg/dl ($p = 0.250$). The Dbilirubin level in a patient in the PI group who was coinfected with HCV besides HIV/HBV as well was −27.4 mg/dl. Excluding this single outlier, the mean Dbilirubin level was significantly different between the PI and non-PI groups (mean Dbilirubin level 0.8 vs. −0.2; $p = 0.01$).

Discussion

We have summarized here the data from our comprehensive survey of HIV/HBV coinfection in Japan, focusing particularly on the clinical features of the patients and the effect of ART on liver function. As we reported earlier, HIV/HBV coinfection was observed in 6.3 % of Japanese HIV-positive patients [7]. Certain considerations for HBV coinfection are important in HIV patient care.

The major transmission route of HIV was male homosexual contact, which accounted for the infection in about 80 % of the patients; thus, male patients were the majority in the present cohort. The most frequently found genotype of HBV was genotype A, which is infrequent in HIV-negative patients in Japan. Genotype A is often found in the United States, Europe, India, and the west coast of Sub-Saharan Africa [25]. Although the data on HBV subgenotypes were not available in our study, some reports showed that most genotype A strains detected in HIV/HBV-coinfected individuals are of genotype Ae [26]. These findings suggest that HBV infection among Japanese HIV carriers is not caused by the spread of indigenous HBV, such as transmission in the perinatal period, but rather specific strains are circulating among the homosexual population in Japan. Genotypes B and C accounted for more than 96 % of the entire Japanese chronic HBV infection [27, 28]. These findings are compatible with the report that the presumed transmission route of HBV in HIV/HBV-coinfected patients is not from Japanese female partners but from male partners, as shown by Koibuchi et al. [29].

Seventy-five percent of HIV/HBV-coinfected patients received ART with two agents against HBV, and its efficacy against HBV as well as HIV is considered to be high. As recommended by the United States Department of Health and Human Services (DHHS) and the Japanese guidelines on HIV treatment, the initiation of ART with NRTIs with anti-HBV activity as the backbone is indicated for HIV/HBV-coinfected patients regardless of HIV viral load or CD4+ T lymphocyte count [30]. Nucleoside analogues can improve liver function in HBV-monoinfected patients [31]. Our study shows that ART decreased the levels of ALT and albumin in HIV/HBV-coinfected patients. It is noteworthy that the regimen used in ART includes multiple drugs with anti-HBV activity such as lamivudine plus abacavir, which is unusual for HBV-monoinfected patients.

When we compared the characteristics of patients on ART with those not on ART, there were some notable differences in their immune status and liver function. At the beginning of the observation period, patients on ART showed a lower CD4+ T-cell count and poorer liver function. Our study is a retrospective observation, and patients were not grouped randomly. These observations are rather understandable because those who had a low CD4+ T cell count were more likely candidates for ART. Additionally, patients on ART had a longer observation period and were more likely to develop AIDS. These findings are also understandable because the longer the duration of HIV infection, the more likely is the immune system of the patient to deteriorate. Moreover, once ART is started, patients need to visit clinics or hospitals regularly for a long period; in reality, for the rest of their life. Following current recommendations for the initiation of ART for HIV infection, patients with worse immune status are more likely to receive the treatment. These findings can explain our observation.

Our data show that the serum albumin level and platelet count improved in the patients who were on ART. As the regimen of ART usually contains two drugs against HBV, ART suppresses HBV replication, which may lead to an improved liver function, as observed in HBV-monoinfected patients treated with nucleoside analogues [31]. Long-term treatment with lamivudine was shown to regress the fibrosis of the liver [32, 33] and decrease the proportion of patients with hepatocellular carcinoma complication [34]. In view of these findings, ART for HIV/HBV-coinfected patients may markedly improve the prognosis of patients. In our study, only a small number of patients with advanced liver diseases associated with HBV infection such as cirrhosis or hepatocellular carcinoma were observed, which could be attributable in part to the short observation period and the short duration of HBV infection. If we had a longer observational period, we would be able to clarify the difference in clinical course between the ART and non-ART groups, and the actual significance of ART for HIV/HBV-coinfected patients should become clearer.

We found that some parameters related to liver function changed paradoxically, particularly in the ART group. Although the mean serum albumin level, ALT level, and platelet count improved, the mean serum bilirubin level worsened, from 0.5 to 0.9 mg/dl. On the other hand, the serum bilirubin level in the non-ART group decreased. Both changes are statistically significant, which suggests

that the observed hyperbilirubinemia was not associated with HBV activity. The increase in serum bilirubin level is presumably caused by PIs. Hyperbilirubinemia following PI administration was previously reported [35]. Although it is unclear whether hyperbilirubinemia itself may lead to liver injury, PIs should be used carefully particularly for patients with advanced liver diseases.

Our present study has one major limitation; that is, the effect of alcohol on liver function was not analyzed because the history of alcohol consumption could not be obtained in the majority of the studied patients. Excessive alcohol consumption has been found to be an important risk factor for the development of severe hepatic injury in HIV-infected patients with [3] or without HCV coinfection [5]. Our present study showed that among the 26 patients whose history of alcohol consumption was available, only 2 patients were habitual drinkers. The results suggested that the effect of alcohol on liver function is small in HIV/HBV-coinfected patients in Japan.

In conclusion, ART with anti-HBV drugs may retard the progression of liver diseases and prevent liver-related death in HIV/HBV-coinfected patients. Multiple agents with anti-HBV activity seem essential for the efficacy. PIs should be carefully used particularly for patients with advanced liver diseases.

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Research Article

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Comparison of resection and ablation for hepatocellular carcinoma: A cohort study based on a Japanese nationwide survey

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Background & Aims: The treatment of choice for early or moderately advanced hepatocellular carcinoma (HCC) with good liver function remains controversial. We evaluated the therapeutic impacts of surgical resection (SR), percutaneous ethanol injection (PEI), and radiofrequency ablation (RFA) on long-term outcomes in patients with HCC.

Methods: A database constructed on the basis of a Japanese nationwide survey of 28,510 patients with HCC treated by SR, PEI, or RFA between 2000 and 2005 was used to identify 12,968 patients who had no more than 3 tumors (≤3 cm) and liver damage of class A or B. The patients were divided into SR (n = 5361), RFA (n = 5548), and PEI groups (n = 2059). Overall survival and time to recurrence were compared among them.

Results: Median follow-up was 2.16 years. Overall survival at 3 and 5 years was respectively 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA, and 78.9%/56.3% in the PEI. Time to recurrence at 3 and 5 years was 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively. On multivariate analysis, the hazard ratio for death was significantly lower in the SR group than in the RFA (SR *vs.* RFA:0.84, 95% confidence interval, 0.74–0.95; $p = 0.006$) and PEI groups (SR *vs.* PEI:0.75, 0.64–0.86; $p = 0.0001$). The hazard ratios for recurrence were also lower in the SR group than in the RFA (SR *vs.* RFA:0.74, 0.68–0.79; $p = 0.0001$) and PEI groups (SR *vs.* PEI:0.59, 0.54–0.65; $p = 0.0001$).

Conclusions: Our findings suggest that surgical resection results in longer overall survival and shorter time to recurrence than either RFA or PEI in patients with HCC.

Keywords: Hepatectomy; Surgical resection; Radiofrequency ablation; Percutaneous ethanol injection.

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Abbreviations: HCC, hepatocellular carcinoma; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transcatheter hepatic arterial chemoembolization.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women, worldwide [1]. Outcomes remain disappointing, despite recent progress in the techniques of diagnosis and therapy. Japanese [2], European [3] and American [4] clinical practice guidelines strongly recommend surgical resection (SR) and percutaneous ablation, including radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI), for the management of early or moderately advanced HCC (i.e., up to 3 tumors 3 cm or less in diameter) in patients with adequately maintained liver function. Although comparative studies of these treatments have been conducted previously [5–7], the most suitable treatment strategy still remains controversial.

By nationwide surveys initiated in 1965, the Liver Cancer Study Group of Japan has prospectively collected data on patients with HCC in Japan. The Group conducted two retrospective analyses to define the treatment with the best outcomes [8,9]. However, each of the analyses was flawed, and had several problems: data on RFA were not included in the first report [8], and the follow-up period was short in the second one [9]. Although the second analysis demonstrated that surgical resection was superior to RFA and PEI for preventing recurrence [9], no apparent difference in the overall survival could be discerned between surgery and percutaneous ablation therapies (RFA and PEI). Thus, the treatment of choice for less advanced HCC still remains under debate.

Before starting this study, the results of 2 randomized controlled trials (RCT) were available [10,11]. As we pointed out in a previous report [12], however, the study designs of these 2

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trials were critically flawed by factors such as insufficient sample size, excessively optimistic hypotheses, and high conversion ratios. Because of these problems, the results of the two RCTs do not allow firm conclusions to be drawn concerning the important clinical question: is surgery or percutaneous ablation the treatment of choice for early or moderately advanced HCC? To answer this question, we conducted this cohort study based on the latest data available from a Japanese nationwide survey.

Patients and methods

Patients and settings

The Liver Cancer Study Group of Japan has performed nationwide surveys of patients with primary liver cancer since 1965. Patients are registered and followed up, as reported previously [9]. Although this study protocol was not submitted to the Institutional Review Board of each institution participating in the nationwide survey, the collection and registration of data of patients with HCC were performed with the approval of each institution. Because RFA has been available for clinical use since 1999 in Japan, we set the study period from 2000 to 2005, to exclude preliminary experiences with RFA. During this period, a total of 28,510 patients with HCC were registered and received surgical resection, RFA or PEI as the primary treatment with curative intent for HCC. We identified 12,968 patients who met the following criteria: (1) liver function classified as liver damage A or B defined by the Liver cancer Study Group of Japan [13]; (2) number of tumors 3 or less; (3) maximum tumor diameter $\leqslant$3 cm. The 12,968 patients were divided into 3 groups according to the treatment received: SR group (n = 5361, 41.3%), RFA group (n = 5548, 42.8%), and PEI group (n = 2059, 15.9%). The diagnostic criteria and details of follow-up were described previously [8]. Because it has been unusual for biopsies to be performed in cases treated by percutaneous ablation in Japan, histological findings such as microscopic vascular invasion, tumor grading, and microscopic intrahepatic metastasis were not evaluated in this study. Relevant clinical data were collected and analyzed.

Statistical analyses

The baseline characteristics of the three groups (Table 1) were compared by analysis of variance for continuous variables and by Chi-square or Mantel-trend tests for categorical variables. Consistent with our preliminary report [9], the SR group had a higher proportion of younger patients and male patients than the RFA and PEI groups. Hepatitis C virus infection was less prevalent in the SR group than in the RFA and PEI groups. Based on the liver damage class, serum albumin and total bilirubin levels, platelet counts, and the indocyanine green retention rate at 15 min, liver function was better in the SR group than in the RFA and PEI groups, consistent with our previous report [9]. As for tumor-related factors, the number of tumors was smaller, and the maximum tumor diameter was larger in the SR group than in the RFA or PEI group. The SR group had the lowest proportion of patients with abnormally elevated alpha-fetoprotein levels ($\geqslant$15 ng/ml) and the highest proportion of patients with abnormally elevated des-γ-carboxy prothrombin levels ($\geqslant$40 AU/ml).

Overall survival and time to recurrence curves were plotted using the Kaplan–Meier method and compared with the use of the log-rank test. Recurrence was diagnosed on the basis of imaging studies, clinical data, and/or histopathological studies at each institution [9].

The therapeutic impacts of surgical resection, RFA and PEI were estimated using a Cox proportional hazards model including the following 10 covariates: age, gender, liver damage class, hepatitis C virus antibody, hepatitis B surface antigen, platelet count, number of tumors, tumor size, and serum alpha-fetoprotein and des-γ-carboxy prothrombin levels. The results of multivariate analysis were expressed as hazard ratios with 95% confidence intervals. p values of <0.05 were considered to indicate statistical significance.

For the subgroup analyses, the study populations were classified into 8 subgroups according to the tumor size (< or $\geqslant$2 cm), tumor number (single or multiple), and liver damage class (A or B). Macroscopic vascular invasion was excluded from the subgroup analyses because its presence is a contraindication to percutaneous ablation therapies. The therapeutic impacts of the three treatments were evaluated in each of these subgroups, and hazard ratios with 95% confidence intervals and p values were calculated according to the above three factors (tumor size, number of tumors, and liver damage class).

Results

The median follow-up after treatment was 2.16 years, and the 5th and 95th percentiles were 0.14 and 5.19 years, respectively. The overall survival rates at 3/5 years were 85.3%/71.1% in the SR group, 81.0%/61.1% in the RFA group, and 78.9%/56.3% in the PEI group (Fig. 1). The median survival times were 8.4, 5.9, and 5.6 years in the three groups, respectively. The time to recurrence rates at 3/5 years in the 3 groups were 43.3%/63.8%, 57.2%/71.7%, and 64.3%/76.9%, respectively (Fig. 2).

According to the results of the multivariate analysis, the hazard ratio for death in the SR group was 0.84 (0.74–0.95, p = 0.006) relative to that in the RFA group, and 0.75 (0.64–0.86, p = 0.0001) relative to that in the PEI group (Table 2A). The hazard ratios for recurrence in the SR group were 0.74 (0.68–0.79, p = 0.0001) and 0.59 (0.54–0.65, p = 0.0001) relative to those in the RFA and PEI groups, respectively (Table 2B). These results indicated that the overall survival and time to recurrence rates were both significantly better in the SR group than in the RFA and PEI groups.

The overall survival rates following surgical resection, RFA and PEI in the 4 subgroups with a single tumor are shown in Fig. 3A–D. The results of the subgroup analyses (summarized in Fig. 4A) showed that the overall survival was significantly longer in the SR group than in the RFA group in 2 subgroups of patients, namely, those who had a single tumor smaller than 2 cm in diameter with liver damage class A, and those who had a single tumor 2 cm or larger in diameter with liver damage class B.

As shown in Fig. 4B, the time to recurrence was shorter in the SR group than that in the RFA group in the 4 following subgroups: patients with a single tumor with liver damage class A (regardless of the tumor size), those with multiple tumors 2 cm or larger in diameter with liver damage class A, and those with a single tumor 2 cm or larger in diameter with liver damage class B.

Discussion

Our study showed that surgical resection was associated with significantly lower risk of both death and recurrence as compared to RFA and PEI in patients with early or moderately advanced HCC. Our previous preliminary report [9] suggested that surgery reduces the risk of recurrence, but failed to demonstrate any difference in the overall survival between surgery and percutaneous ablation therapies in patients with early or moderately advanced HCC. The present study reconfirms that surgery is associated with a reduced recurrence rate and newly shows that surgery yields a longer overall survival than percutaneous ablation therapies.

Differences in the results between the present study and previous investigations are most likely related to the sample size and length of follow-up. The total number of subjects increased markedly from 7185 in our previous study to 12,968 in this study, and the median follow-up period increased from 10.4 months to 2.16 years (25.9 months). These factors are considered not only to have enhanced the reliability of our findings, but also to have strengthened our conclusions. We believe that our results, which are, of course, subject to the inherent drawbacks of the study design, are meaningful, given the current lack of credible data derived from well-designed RCTs.

The large sample size and prolonged follow-up period also allowed us to perform several subgroup analyses, which were not feasible in our previous study [9]. We classified the patients

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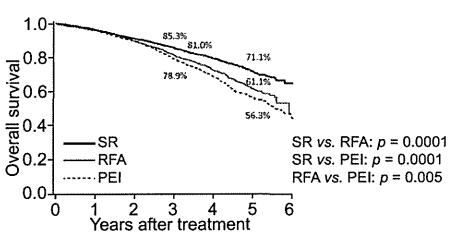
Table 1. Baseline characteristics.

Variables	SR n = 5361	RFA n = 5548	PEI n = 2059	p value
Age, median (5, 95 percentile), yr	66 (48, 77)	69 (52, 80)	69 (52, 80)	<0.0001[a]
Sex				<0.0001[b]
Male, No. (%)	3967 (74.0)	3569 (64.3)	1303 (63.3)	
Female, No. (%)	1394 (26.0)	1979 (35.7)	756 (36.7)	
Hepatitis virus infection				<0.0001[b]
HBs Ag(+)/HCV-Ab(-), No. (%)	908 (16.9)	462 (8.3)	141 (6.8)	
HBs Ag(-)/HCV-Ab(+), No. (%)	3393 (63.3)	4263 (76.8)	1632 (79.3)	
HBs Ag(+)/HCV-Ab(+), No. (%)	106 (2.0)	87 (1.6)	32 (1.6)	
HBs Ag(-)/HCV-Ab(-), No. (%)	760 (14.2)	512 (9.2)	160 (7.8)	
Unknown	194 (3.6)	224 (4.0)	94 (4.6)	
Liver damage				<0.0001[b]
A, No. (%)	4000 (74.6)	3349 (60.4)	1204 (58.5)	
B, No. (%)	1361 (25.4)	2199 (39.6)	855 (41.5)	
Serum albumin, median (5, 95 percentile), g/dl	3.9 (3.1, 4.6)	3.7 (2.9, 4.4)	3.7 (2.8, 4.4)	<0.0001[a]
Serum total bilirubin, median (5, 95 percentile), mg/dl	0.8 (0.4, 1.5)	0.9 (0.4, 1.9)	0.9 (0.4, 2.2)	<0.0001[a]
Platelet count, median (5, 95 percentile), x 10^4/μl	12.6 (5.8, 24.0)	9.9 (4.5, 20.4)	9.5 (4.4, 19.6)	<0.0001[a]
ICG R15, median (5, 95 percentile), %	15 (5, 35)	22 (7, 51)	24 (8, 51)	<0.0001[a]
Tumor number				<0.0001[c]
Single, No. (%)	4458 (83.2)	4068 (73.3)	1449 (70.4)	
Two, No. (%)	706 (13.2)	1096 (19.8)	443 (21.5)	
Three, No. (%)	197 (3.7)	384 (6.9)	167 (8.1)	
Tumor size, median (5, 95 percentile), mm	23 (12, 30)	20 (10, 30)	17 (10, 30)	<0.0001[a]
Alpha-fetoprotein				<0.0001[b]
≥15 ng/ml, No. (%)	2726 (50.9)	3028 (54.6)	1125 (54.6)	
<15 ng/ml, No. (%)	2457 (45.8)	2301 (41.5)	828 (40.2)	
Unknown, No. (%)	178 (3.3)	219 (3.9)	106 (5.2)	
Des-γ-carboxy prothrombin				<0.0001[b]
≥40 AU/ml, No. (%)	2182 (40.7)	1593 (28.7)	541 (26.3)	
<40 AU/ml, No. (%)	2651 (49.5)	3322 (59.9)	1169 (56.8)	
Unknown, No. (%)	528 (9.9)	633 (11.4)	349 (17.0)	

HBsAg, hepatitis B virus antigen; HCV-Ab, hepatitis C virus antibody; ICG R15, indocyanine green retention rate at 15 min.
[a]ANOVA.
[b]Chi-square.
[c]Mante-trend test.



Patients at risk							
SR	5361	3833	2570	1680	894	400	29
RFA	5548	3780	2328	1264	569	160	5
PEI	2059	1595	1112	718	444	247	58

Fig. 1. Overall survival curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

into 8 subgroups according to 3 factors (liver damage class, tumor size, and number of tumors), which have repeatedly been shown to be clinically relevant prognostic factors. The results of the subgroup analyses indicated that surgical resection would effectively prevent recurrence in patients with relatively advanced HCC (2–3 cm in diameter) among the study populations, irrespective of liver damage class or number of tumors. This finding suggests that surgery might be superior to percutaneous ablation therapies in patients with a more advanced tumor stage. As for the subgroups with a single tumor, surgical resection yielded better overall survival and time to recurrence rates than RFA or PEI. Especially in the subgroup with a single tumor smaller than 2 cm in diameter, both the overall and time to recurrence rates were statistically significantly better after surgery than after RFA, whereas no such statistically significant differences in these two parameters between the two treatment groups were detected in a few subgroups with a single tumor, maybe due to the insufficient sample size of the subgroups. Thus, surgical resection would be considered as the treatment modality of first choice for a single HCC, as recommended by the Japanese clinical practice guideline [2]. Overall, there was a trend toward superior

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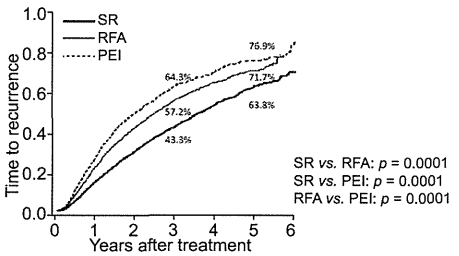

Patients at risk							
SR	5361	3265	1844	1039	451	189	15
RFA	5548	2954	1396	591	225	62	4
PEI	2059	1154	583	304	172	90	15

Fig. 2. Time to recurrence curves after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI).

overall and time to recurrence rates after surgery than after RFA and PEI.

The reason why the long-term outcomes of the SR group were better than those of the PEI and RFA groups cannot be definitely clarified from the results of this study, however, in theory, surgical resection has the advantage of offering better local control of HCC over PEI and RFA, both of which have some potential risks of local recurrence associated with insufficient ablation. In addition, anatomic resection to remove minute tumor satellites [14] might have decreased the recurrence rate in the SR group, although this remains a speculation.

Recently, the latest trial from China [15], which had an adequate sample size (total 230 patients), reported that surgical resection yielded significantly better long-term outcomes than RFA. Although the study design was better than that of the two previously reported RCTs [10,11], it appeared to have limitations with respect to the results, such as drop in the overall survival in the RFA group as compared with that in the surgery group during the early period after treatment. The early deaths in the RFA group could have been treatment-related rather than cancer-related. Thus, no conclusion can be drawn from the three currently available RCTs.

One of the limitations of our study is the diversity of demographic factors in the study population, which would have been

Table 2. Hazard ratios for death and recurrence adjusted by multivariate analysis.

A For death

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.84	0.74, 0.95	0.006
	SR *vs.* PEI	0.75	0.64, 0.86	0.0001
	RFA *vs.* PEI	0.88	0.77, 1.01	0.08
Age	<65 *vs.* ≥65	0.71	0.63, 0.79	0.0001
Sex	Female *vs.* male	0.87	0.78, 0.98	0.03
HBsAg	Positive *vs.* negative	0.91	0.74, 1.11	0.34
HCV Ab	Positive *vs.* negative	0.93	0.79, 1.10	0.40
Liver damage	A *vs.* B	0.62	0.56, 0.69	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4$/μl	0.76	0.68, 0.85	0.0001
Tumor size	<2 *vs.* ≥2 cm	0.82	0.73, 0.92	0.0007
Tumor number	Single *vs.* multiple	0.72	0.64, 0.80	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.66	0.59, 0.74	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.59	0.53, 0.66	0.0001

B For recurrence

Variables		Hazard ratio	95% CI	*p* value
Treatments	SR *vs.* RFA	0.74	0.68, 0.79	0.0001
	SR *vs.* PEI	0.59	0.54, 0.65	0.0001
	RFA *vs.* PEI	0.81	0.74, 0.88	0.0001
Age	<65 *vs.* ≥65	0.83	0.78, 0.89	0.0001
Sex	Female *vs.* male	0.88	0.82, 0.95	0.0001
HBsAg	Positive *vs.* negative	1.04	0.92, 1.17	0.53
HCV Ab	Positive *vs.* negative	1.15	1.04, 1.27	0.007
Liver damage	A *vs.* B	0.87	0.81, 0.93	0.0001
Platelet count	$\geq 10^4$ *vs.* $<10^4$/μl	0.92	0.86, 0.98	0.02
Tumor size	<2 *vs.* ≥2 cm	0.84	0.79, 0.90	0.0001
Tumor number	Single *vs.* multiple	0.69	0.64, 0.74	0.0001
AFP	<15 *vs.* ≥15 ng/ml	0.71	0.67, 0.76	0.0001
DCP	<40 *vs.* ≥40 AU/ml	0.72	0.67, 0.77	0.0001

HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; Ab, antibody; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; SR, surgical resection; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; CI, confidence interval.

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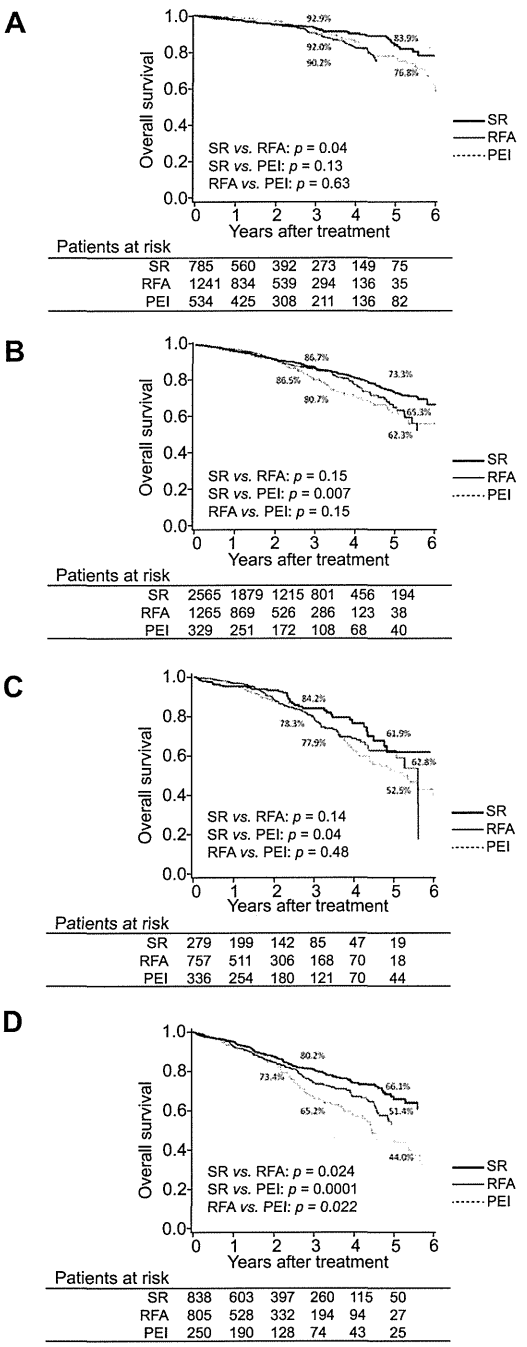

Fig. 3. Overall survival rates after surgical resection (SR), radiofrequency ablation (RFA), and percutaneous ethanol injection (PEI) in the subgroup of cases with single tumor and liver damage class A and B. (A) Liver damage class A, a single tumor (<2 cm); (B) liver damage class A, a single tumor (2–3 cm); (C) liver damage class B, a single tumor (<2 cm); (D) liver damage class B, a single tumor (2–3 cm).

caused by the selection process of treatment modalities. As similar to the previous retrospective studies [5–9], the patients amenable to surgery had had younger age, less prevalence of hepatitis

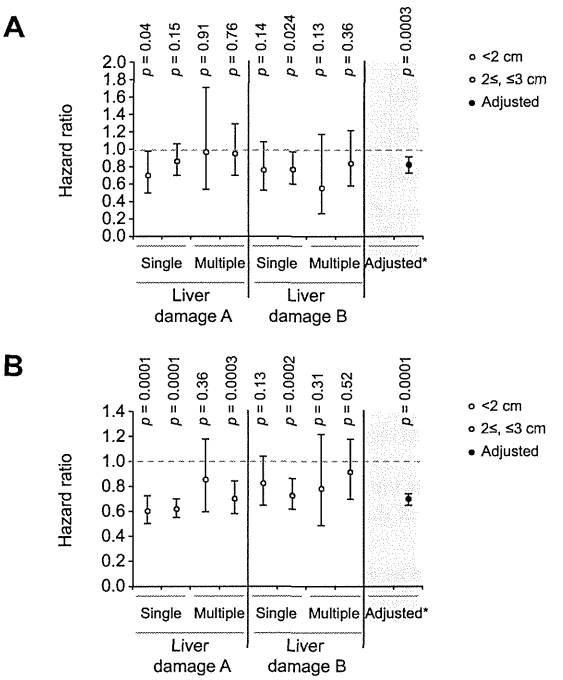

Fig. 4. Hazard ratios for death and recurrence with 95% confidence intervals and *p* values after surgical resection relative to those after radiofrequency ablation in the 8 subgroups. *The adjusted values for death and recurrence were calculated according to the three factors (tumor size, number of tumors, and liver damage class), as done in each subgroup. (A) Hazard ratios for death; (B) hazard ratios for recurrence.

C virus infection, better liver function, less association with portal hypertension, fewer number of tumors and lower alpha-fetoprotein level, whereas their tumor size was larger and their des-γ-carboxy prothrombin level was higher. To minimize potential effects of confounding factors, we studied patients who had similar tumor-related and liver function-related factors and performed multivariate analysis using 10 clinically important factors, similar to our previous study [9]. Although it is impossible to completely eliminate potential negative impacts of demographic diversity, we believe that our results are clinically meaningful, because of the large sample size of our study. In Japan, a nationwide RCT in patients with HCC is now ongoing, and the results are expected to lead to more definitive conclusions [16].

Another potential limitation of our study is the lack of data on liver function during the follow-up, which precluded assessment of the relationship between the liver function status and the choice of treatment at recurrence. In HCC, the influence of the first treatment is considered to be smaller than that in other primary malignant diseases, because the liver function remarkably affects the recurrence rate. Further investigations, particularly prospective clinical trials, are needed to address these issues.

In conclusion, this large cohort study based on data obtained by a nationwide survey in Japan, suggests that surgical resection may offer some advantage over RFA and PEI in terms of both overall survival and time to recurrence in patients with less advanced HCC. Although our results are considered as being more reliable than those of previous studies comparing the treatment

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outcomes in HCC, our conclusions need to be confirmed by future RCTs.

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Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Article

Coexpression network analysis in chronic hepatitis B and C hepatic lesions reveals distinct patterns of disease progression to hepatocellular carcinoma

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Chronic infections with the hepatitis B virus (HBV) and hepatitis C virus (HCV) are the major risks of hepatocellular carcinoma (HCC), and great efforts have been made towards the understanding of the different mechanisms that link the viral infection of hepatic lesions to HCC development. In this work, we developed a novel framework to identify distinct patterns of gene coexpression networks and inflammation-related modules from genome-scale microarray data upon viral infection, and further classified them into oncogenic and dysfunctional ones. The core of our framework lies in the comparative study on viral infection modules across different disease stages and disease types—the module preservation during disease progression is evaluated according to the change of network connectivity in different stages, while the similarity and difference in HBV and HCV are evaluated by comparing the overlap of gene compositions and functional annotations in HBV and HCV modules. In particular, we revealed two types of driving modules related to infection for carcinogenesis in HBV and HCV, respectively, i.e. pro-apoptosis modules that are oncogenic in HBV, and anti-apoptosis and inflammation modules that are oncogenic in HCV, which are in concordance with the results of previous differential expression-based approaches. Moreover, we found that intracellular protein transmembrane transportation and the transmembrane receptor protein tyrosine kinase signaling pathway act as oncogenic factors in HBV-HCC. Our findings provide novel insights into viral hepatocarcinogenesis and disease progression, and also demonstrate the advantages of an integrative and comparative network analysis over the existing differential expression-based approach and virus–host interactome-based approach.

Keywords: gene coexpression network, hepatitis B and C virus, hepatocellular carcinoma, disease progression, systems biology

Introduction

It has been estimated that chronic infections with the hepatitis B virus (HBV) and hepatitis C virus (HCV) account for up to 80% of hepatocellular carcinoma (HCC; Perz et al., 2006). Although chronic hepatitis caused by HBV and HCV is hardly distinguished by histological examination or clinical manifestations, the virological features of HBV and HCV are obviously different. HBV is a DNA virus that can be transported into the nucleus and integrated into the host DNA, thus directly transforming hepatocytes. In contrast, HCV is an RNA virus that replicates in the cytoplasm and is unable to integrate into the host genome (Tsai and Chung, 2010; Bouchard and Navas-Martin, 2011). Ever since the discovery of these two viruses, great efforts have been made towards the understanding of the molecular events and cellular signal transduction pathways that are altered by HBV and HCV infections (Iizuka et al., 2002; Honda et al., 2006; Mas et al., 2009; Ura et al., 2009), as well as the mechanisms that link HBV or HCV infections and hepatic lesions to HCC development (Wurmbach et al., 2007; Mas et al., 2009). Studies in this area include comparisons of microarray gene/microRNAs expression in HBV-HCC and HCV-HCC, identification of significantly differentially expressed genes/microRNAs under the two types of HCC, and analysis of functional annotations represented by them. It was reported that inflammation, anti-apoptosis, immune response, cell cycle and lipid metabolism were predominant in HCV, but pro-apoptosis, DNA damage and DNA repair response were predominant in HBV (Iizuka et al., 2002; Honda et al., 2006; Ura et al., 2009). There is also research (Wurmbach et al., 2007; Mas et al., 2009) focusing on a stepwise carcinogenic process from normal liver to HCV cirrhosis to HCV-HCC, or from preneoplastic lesions (cirrhosis and dysplasia) to HCV-HCC, and a positive trend was found in MHC class-I receptor activity, DNA damage checkpoint cell division and ubiquitin cycle genes

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during this process (Mas et al., 2009). Although these efforts have suggested different oncogenic factors in HBV and HCV, as well as marker pathways during HCV-HCC progression, an integrative and comparative study of gene expression profiles in both HBV-HCC and HCV-HCC progression has yet to be conducted.

Network-based systems biology approaches (Liu et al., 2012) typically involve identification of groups of genes or network modules by microarray data analysis, whose expression levels are highly correlated across samples (Stuart et al., 2003; Zhang and Horvath, 2005; Oldham et al., 2008; Dewey et al., 2011). Such holistic approaches have several advantages over standard methods such as differential expression analysis, whose result is usually a list of genes, each of which is deemed significant in isolation (Chen et al., 2009, 2012). Actually, quantitative assessment of module preservation in different phenotypes using both gene expression and network connectivity as summation (Miller et al., 2010; Dewey et al., 2011) provides a new avenue in understanding of molecular differences that distinguish functional processes in disease progression (Oldham et al., 2008; Miller et al., 2010).

In this work, we developed a new framework to study the differences and similarities in HBV-HCC and HCV-HCC at a network level by an integrative and comparative analysis of weighted gene coexpression modules or networks in HBV-infected and HCV-infected liver tissues. We hypothesized that viral infection is an important stage or factor in carcinogenic progression (Tsai and Chung, 2010; Bouchard and Navas-Martin, 2011), and thus focused on the analysis of viral infection modules, e.g. oncogenic modules and dysfunctional modules. Using this approach, we identified distinct network modules of coexpressed genes with clear functional interpretations in HBV and HCV, as well as their implications of HCC development. We found that pro-apoptosis modules are oncogenic in HBV, but anti-apoptosis and inflammation modules are oncogenic in HCV, which is in concordance with previous differential expression-based approaches. Clearly, these modules are the driving force of carcinogenesis in HBV and HCV, respectively, which cannot be revealed by viral target analysis. In addition, we observed that intracellular protein transmembrane transportation and the transmembrane receptor protein tyrosine kinase signaling pathway were top enriched in HBV oncogenic modules, while a similar process of endosome to lysosome transport was observed in HCV dysfunctional modules. Those results are consistent with the existing knowledge that HCV enters hepatocytes via endocytosis (Bouchard and Navas-Martin, 2011). Although the entry mechanism of uncoated HBV into hepatocytes, and the transport of the viral genome into the nucleus of the host remain unclear (Seeger et al., 2007), the oncogenic modules identified by our approach show their important dysfunctions for HBV-HCC, and this can be a promising topic of future experimental research. Besides comparing the functional annotations of the top-ranked modules, we further identified the module overlap in HBV and HCV and found that the modules of HBV and HCV shared a significant overlap with each other. It implies that these subsets of genes are consistently coexpressed upon both HBV and HCV infection, but they result in the different network topologies and wiring that lead to contrasting functional performances. Last but not least, curating HBV/HCV protein targets (de Chassey et al., 2008; Wu et al., 2010) from literature research and combining them with our analysis result, we provided different viral targets as a potential root cause of these distinctions between HBV-HCC and HCV-HCC. Clearly, these new findings not only demonstrate the effectiveness of our network-based approach on analyzing the complex diseases, but also provide biological insights into viral hepatocarcinogenesis and disease progression.

Results

Overview of our framework

Figures 1 and 2 show the overview of our framework. Coexpression network reconstruction from high-throughput data are illustrated in Figure 1A. Module identification and functional analysis are summarized in Figure 1B, and module analysis for four types of viral infection modules is summarized in Figure 2. This paper focuses on the analysis of viral infection modules in disease progression. After we built gene coexpression networks for HBV and HCV, we identified their coexpresssion modules individually. After we validated their reproducibility in the independent datasets, we filtered out inflammation-related modules upon

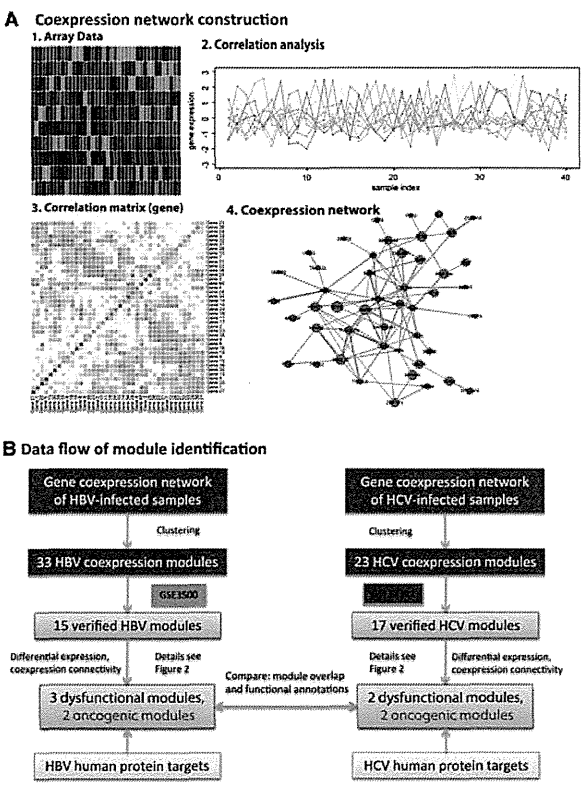
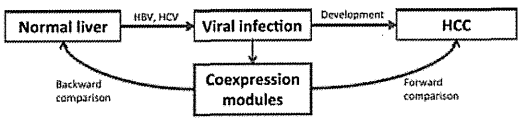


Figure 1 Overview of the framework. (**A**) Gene coexpression network reconstruction. (i) Microarray data filtering and prepossessing (rows correspond to samples and columns correspond to genes). (ii) Correlation analysis of individual genes expression across different samples. (iii) Construction of Pearson's correlation matrix and transformation into a matrix of connection strength. (iv) Coexpression network is established using hierarchical average linkage clustering (WGCNA). (**B**) Framework of module identification and analysis. The details of descriptions can be found in Materials and methods.



	Normal	Viral infection	HCC
Oncogenic	N	Y	Y
Dysfunctional	Y	Y	N
Housekeeping	Y	Y	Y
Transit	N	Y	N

Figure 2 Viral infection modules and their classification. This figure shows how to identify four types of viral infection modules (i.e. oncogenic, dysfunctional, housekeeping, and transit modules). The top subfigure shows the progression of HCC (i.e. from normal liver to viral infection and to HCC), and module comparison centered on viral infection or inflammation stage. The verified coexpression module of viral infection of HBV and HCV is classified into one type of 'oncogenic', 'dysfunctional', 'housekeeping', and 'transit' individually by backward and forward comparison for module preservation. 'Y' or 'N' represents its preservation 'yes' or 'no' in the three stages of disease progression, respectively. For example, one module ('Y' in viral infection) is identified to be 'oncogenic' when it is preserved in HCC ('Y'), but not in normal status ('N').

viral infections. The comparison of these modules in different disease stages for module preservation results in four types inflammation modules. And the comparison of oncogenic and dysfunctional modules in HBV and HCV provides evidence of the similarities and differences in the viral infections. We also tried to investigate their similarities and differences by analyzing the virus–host interactions of humans. The detailed descriptions of our framework are given in Materials and methods.

Constructing gene coexpression networks in HBV- and HCV-infected liver tissues

We set out to investigate the transcriptome upon viral infection and construct gene coexpression networks by applying weighted gene coexpression network analysis (WGCNA) (Zhang and Horvath, 2005). Our study was primarily based on Kanazawa data (Honda et al., 2006; Ivliev et al., 2010), which contains gene expression from 18 normal liver tissues (in normal stage), 36 HBV and 35 HCV-infected liver tissues (in viral-infected or inflammation stage), and different samples of 17 HBV-HCC and 17 HCV-HCC (in HCC stage). The other three datasets were mainly used for validation purposes. Two coexpression networks—one for HBV and the other for HCV—were constructed by calculating the pairwise Pearson's correlation coefficients of gene expressions in 36 HBV-infected samples and 35 HCV-infected samples, respectively. The information about datasets used in the study is shown in Supplementary Table S1. Briefly, the Pearson's correlation matrix for each coexpression network was transformed into a matrix of connection strengths using a power function (power = 6). These connection strengths were then used to calculate the topological overlap (TO), which considers not only the correlation of the two genes, but also the degree of their shared neighbors across the whole network.

Detecting gene coexpression modules in HBV- and HCV-infected liver tissues

Hierarchical average linkage clustering based on TO was used to group genes with highly similar coexpression patterns into modules (Ravasz et al., 2002). For computational reasons, we conducted the network module identification procedure in a blockwise manner with the same parameter setting for all networks. To summarize the scaled gene expression profiles for the identified modules, we used the first singular vector (module eigengene, ME), which is equivalent to the first principle component and explains the largest proportion of the variance of the module genes. We then used the MEs in a procedure to reassign genes to the modules which maximizes the module memberships (see Materials and methods for details). To this end, we identified 33 modules in HBV-infected liver tissues and 23 modules in HCV-infected liver tissues individually (Figure 3A and C), and each of them, containing coordinately expressed genes potentially participated in common cellular processes. The full list of module memberships is provided in Supplementary Table S2.

Identifying viral infection modules that are highly preserved across independent datasets

Because of the different number of gene expression samples and the wide range of coordinate gene regulations (Ivliev et al., 2010), we first validated the identified modules internally by a data-splitting technique in which 70% of the samples were used as a training set (see Materials and methods). After generating 100 such training sets, modules with significant co-clustering statistics (empirical $P < 0.05$) were retained for further validation (Figure 4).

Microarrays are inconsistent for differences in gene expression profiles across datasets and platforms (Wang et al., 2005). To gauge the consistency of our identified modules in independent datasets, two hepatitis virus-infected liver datasets, GSE3500 (Chen et al., 2002, 2004) and GSE14323 (Mas et al., 2009), were assembled. GSE3500 contains 10 samples of normal liver, 33 HBV-infected liver samples and 52 HBV-infected HCC. GSE14323 contains 19 samples of normal liver, 41 HCV-infected liver samples and 55 HCV-infected HCC. Detailed descriptions about these datasets are provided in Supplementary Table S1. We filtered and preprocessed the two datasets, and further identified gene coexpression modules from virus-infected status using the same procedure as described previously. Since the datasets contain different genes, we used the common genes shared by two datasets to compute the significance of the module overlap based on the hypergeometric test (Figure 3B and D). For HCV modules, 21 out of 23 of them have significant overlap ($P < 0.05$) with at least one module derived from GSE14323 providing confidence in the reproducibility of HCV gene coexpression modules. For HBV modules, however, 17 out of 33 of them have significant overlap with at least one module derived from GSE3500. Nevertheless, to ensure the reliability of our study, we identified interested modules that not only pass the internal validation, but also can be reproduced on independent datasets, which eventually resulted in 17 HCV modules and 15 HBV modules. We found that some most important modules—modules that will be classified as oncogenic and dysfunctional modules in the later sections—were not affected by such filtering. These modules represent sets of genes that are presented on and consistently coexpressed in diverse microarray platforms of viral infection.

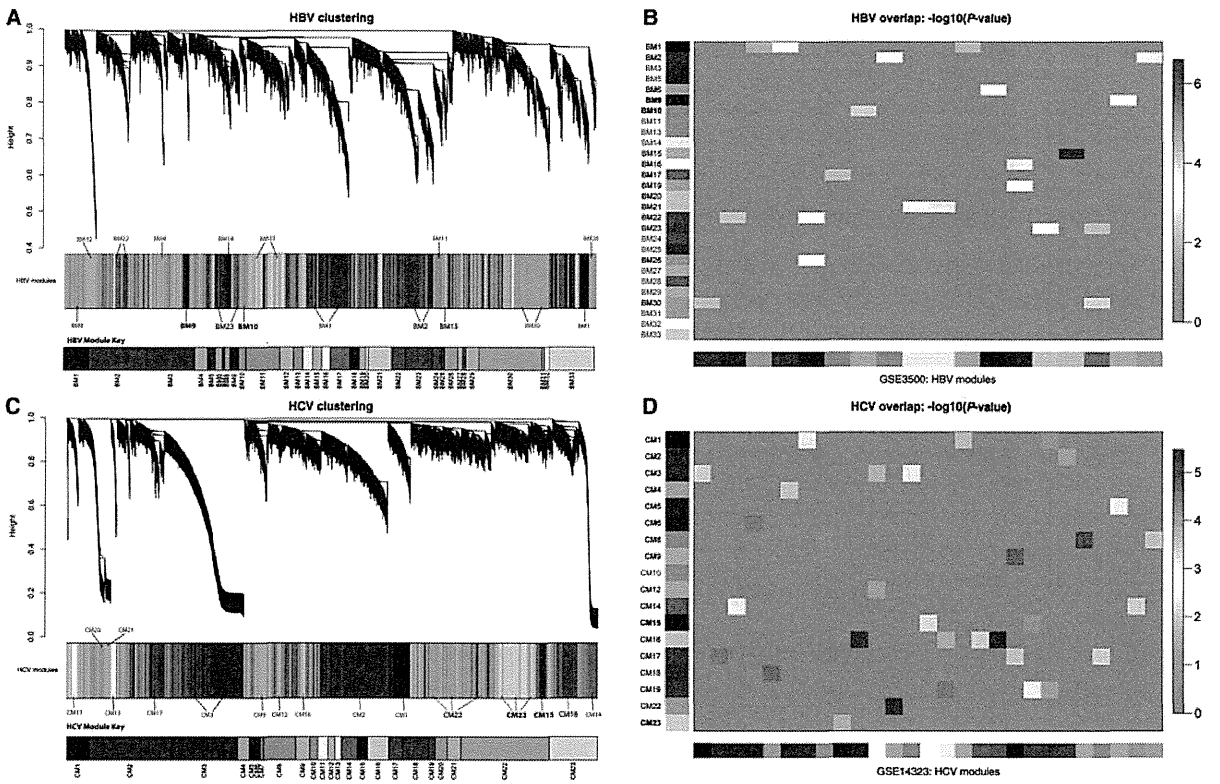


Figure 3 Identification of gene coexpression modules in 36 HBV- and 35 HCV-infected liver tissues and module reproducibility in independent datasets. Hierarchical average linkage clustering was applied to gene–gene adjacencies, which were defined on the basis of TO. Dynamic tree cut algorithm was applied to the dendrogram for module identification, and genes in the same branch can be assigned to different modules. The analysis identified 33 HBV modules (**A**) and 23 HCV modules (**C**) represented by different colors on the horizontal bar. Oncogenic modules (**A**: BM2, BM15, and BM23; **C**: CM18 and CM22) are marked in bold red font and dysfunctional modules (**A**: BM9 and BM10; **C**: CM15 and CM23) are marked in bold black font. In **B** and **D**, vertical modules were identified from our working dataset (Kanazawa data, corresponding to **A** and **C**, respectively), while horizontal modules were identified from independent dataset. Significance of pair-wise module-module overlap was based on Fisher's exact test *P*-values, using module assignment of the common genes shared by two datasets. (**B**) 21 out of 33 HBV modules have at least one significant ($P < 0.001$) overlapping modules in independent dataset (GSE3500). (**D**) 17 out of 23 HCV modules have at least one significant ($P < 0.001$) overlapping modules in independent dataset (GSE14323). Only these reproduced modules were kept for further analysis, and filtered module numbers are marked in grey.

We have validated the reproducibility of our identified gene coexpression modules in independent datasets, and further investigated whether these modules can be used to distinguish different stages of disease progression, reasoning that viral infection is an important transforming stage from normal to HCC (Tsai and Chung, 2010). MEs, i.e. the first singular vector of expressions in the module, were treated as the 'activity' and used to build classifiers for predicting the disease status given a test expression profile. For this purpose, MEs were used as feature values in a classifier based on svmRadial (Alexandros and David, 2006), and the technique of 5-fold cross validation was applied to select the optimal model that maximizes the area under the curve (AUC) of the receiver-operating characteristic. Once the optimal classifier was determined from one dataset, it was used to predict disease status for an independent dataset. Only the 15 HBV modules and 17 HCV modules that passed both internal and external validation were used for classification. Briefly, we trained classifiers on the working Kanazawa dataset and tested them on the validation one, and *vice versa*. To compute MEs on an independent dataset, we mapped gene compositions of each module to the independent dataset and calculated the first singular vector from the new gene expression profiles.

Our working Kanazawa dataset consists of various disease states in HCC progression: 18 normal, 36 HBV-infected, 35 HCV-infected, 17 HBV-HCC and 17 HCV-HCC (Supplementary Table S1). To examine the relationship among five categories of groups, i.e. normal, HBV-liver, HCV-liver, HBV-HCC, HCV-HCC, we built up five binary classifiers: normal and HBV-liver, HBV-liver and HBV-HCC, normal and HCV-liver, HCV-liver and HCV-HCC, HBV-HCC, and HCV-HCC. The final classification performance was defined as the AUC on one dataset using the classifier optimized from the other dataset (Figure 5 and Supplementary Figure S2). It was shown from Figure 5A and B

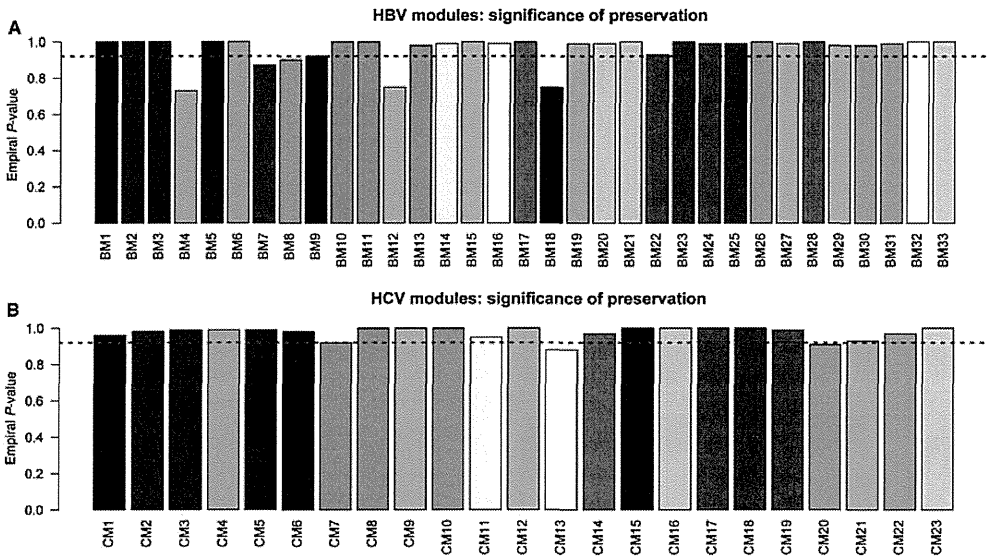


Figure 4 Internal validation of HBV (**A**) and HCV (**B**) modules. Each colored bar corresponds to a module. Red dash line indicates cutoff for statistical significance (empirical $P < 0.05$ or probability value > 0.95). Modules passing the cutoff line represent genes coexpressed in a wide range of samples while modules below the cutoff line represent genes coexpressed in only a subset of samples.

that gene coexpression modules identified from virus-infected status clearly distinguish expression profiles of normal and HCC. The results demonstrate the distinct module-gene expression profiles in different disease stages. However, the modules did not perform so well in classifying the two types of HCC, namely, HBV-HCC and HCV-HCC on the independent dataset GSE19665 (Deng et al., 2010; Supplementary Figure S2). One possibility is that the two types of HCC differ in the case of hepatocarcinogenesis, but they are rather similar at least in terms of the expression profile when cancer has already occurred. The other possibility is that the gene expression profile changes dramatically from viral infection to HCC, rendering it unsuitable to classify HCC types with these modules derived from the stage of viral infection.

Selecting oncogenic and dysfunctional modules related to inflammation

We have identified gene coexpression modules from HBV/HCV-infected liver tissues, i.e. in the viral infection or inflammation stage, validated their reproducibility in independent datasets, and we also discovered the distinct module expression profiles in the three stages of disease progression, i.e. normal, viral infection, and HCC, which could be used for phenotype classification in HBV and HCV, respectively. To focus on small subsets of modules which are most relevant to HCC, we investigated the dynamics of modules during disease progression and selected two types of modules, i.e. oncogenic and dysfunctional modules that are most likely to be related to HCC. As shown in Figure 2, we defined oncogenic and dysfunctional as follows. (i) 'Oncogenic': modules that are formed upon viral infection (i.e. they are disrupted in normal liver tissues) but are preserved in HCC, which represent inflammation-related oncogenic biological processes that are activated only upon viral infection. (ii) 'Dysfunctional': modules that are preserved in normal liver tissues but are disrupted in HCC, which represent tumor suppressive processes that remain effective upon viral infection. There are two more types of modules identified from viral-infected status. (iii) 'Housekeeping': those modules are preserved in both normal tissue and HCC. (iv) 'Transit': those modules are preserved in neither normal nor HCC. The housekeeping modules remain static during disease progression and are more likely to perform essential housekeeping functions, while the transit modules are more likely to be identified only in viral infection. They may be specifically responsive to the viral infection in this critical process and may indicate no disease progression characteristics of HCC. A graphical illustration of the four types of modules is shown in Figure 2. In order to determine which modules and their corresponding dysfunctional processes were activated upon viral infection, we defined two types of changes, i.e. the change in network topology which measures the gene–gene coexpression relationship and in the enrichment of differential expressed (DE) genes which measures the alternation of individual gene expression across phenotypes. We noticed that direct comparison of gene–gene correlation coexpression within modules between disease stages is unsuitable because the sample size in each stage varies. Therefore, we adopted a previously developed measure of the preservation density in intramodular connections between two networks (Dewey et al., 2011), and random permutation was run to assess their significance of preservation density (see Materials and methods). We defined modules with preservation density higher than 95% random permutations as significantly conserved and those with preservation density lower than 95% random permutations (empirical $P < 0.05$) as significantly disrupted (Figure 5C and D, and Table 1). To identify modules with significant differential

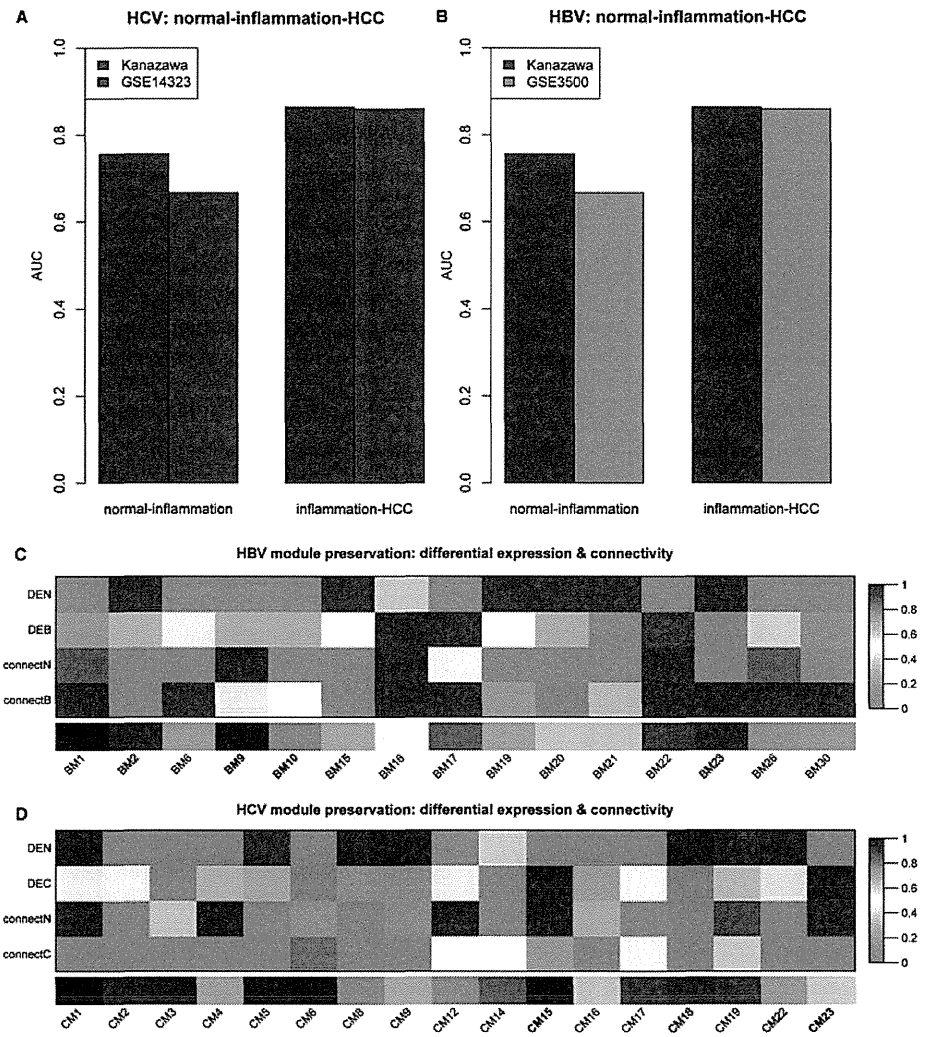


Figure 5 Phenotype classification results of the identified gene coexpression modules and preservation of viral infection modules in different disease stages. The coexpression modules identified from virus-infected inflammation status could distinguish status of normal and HCC (**A** and **B**), indicating the distinct expression profiles in three stages of disease progression, e.g. normal, virus-induced inflammation, and HCC. MEs of the reproduced modules were used as feature values, and svmRadial-based classifiers were trained in one dataset and evaluated in the other dataset, respectively. Preservation of viral infection modules in normal status and HCC (**C** and **D**) was evaluated in terms of differential expression (DEN: differential expression in normal vs HBV/HCV, DEB: differential expression in HBV vs HBV-HCC, DEC: differential expression in HCV vs HCV-HCC) and connectivity (connectN: correlation in normal vs HBV/HCV, connectB: correlation in HBV vs HBV-HCC, connectC: correlation in HCV vs HCV-HCC). The permutation-based score corresponds to the proportion of one thousand permutations in which random gene modules were more preserved (under-representation of differentially expressed genes or enrichment of conserved gene–gene coexpression relationship) than the derived modules. Therefore, red color (score > 0.95) corresponds to highly disrupted modules while green color (score $<$ 0.05) corresponds to highly conserved modules.

expression across phenotypes, we identified differentially expressed genes (adjusted $P < 0.05$), and measured the enrichment in the module using a permutation-based approach (see Materials and methods). The reported empirical *P*-value was equivalent to the proportion of random permutations in which random gene modules of the same size had a greater significance of DE than the module tested (Figure 5C and D, and Table 1). To this end, out of 15 HBV modules and 17 HCV modules, we identified 3 HBV modules and 2 HCV modules as oncogenic modules (italic type in Table 1), and 2 HBV modules and 2 HCV modules as dysfunctional modules (bold black in Table 1).

Comparison of selected HBV and HCV modules

Natural questions following module identification are (i) what are the similarities and differences between HBV and HCV modules? (ii) What are the dysfunctional implications for such similarities and differences for HCC? In this section, we analyzed the overlap between modules and enrichment of functional annotations to answer these questions.

Comparison of module overlap. First, comparisons of gene compositions of HBV and HCV modules based on the Fisher's

Table 1 Inflammation-related oncogenic (italic-type font) and dysfunctional (black font) modules, their top functional annotations and viral targets.

Virus	Cluster_index	Cluster_name	DE_normal_virus	DE_virus_HCC	Normal_virus	Virus_HCC	Category	Top functional annotations	Virus targets
HBV	BM2	***Blue***	**1**	0.324	0	0*	Oncogenic	Positive regulation of apoptosis	AIP,BHMT2,CHEK1,FETUB,HIF1A,MAPK9,MMP2,PTEN, PTGS2,RXRA,SDC4,SKP2,XBP1
	BM9	**darkred**	0*	0.314	0.436	**0.954**	Dysfunctional	Cell motion, positive regulation of apoptosis	PSMA7
	BM10	**darkturquoise**	0.04*	0.304	0.488	**0.96**	Dysfunctional	–	DNAJB1
	BM15	***lightgreen***	**1**	0.494	0.004	0.028*	Oncogenic	–	JAG1
	BM23	***Red***	**0.99**	0.014*	**0.974**	0.914	Oncogenic	Intracellular transport	–
HCV	CM15	**Midnight blue**	0.004*	**0.992**	0.216	0.192	Dysfunctional	Endosome to lysosome transport	H19,LZTS2,SRPX2
	CM18	***Red***	**1**	0.012*	0	0*	Oncogenic	Regulation of cell death	ANKRD12,FBN1,FXYD6,ITGB4,JAG2,JAK2, POU3F2,RUSC2,SSR4,TP53BP2,TP53BP2
	CM22	***Turquoise***	***1***	0.43	0	0*	Oncogenic	Positive regulation of transcription, negative regulation of apoptosis	C16orf7,C7,CANX,CANX,CTSB,FES,GRN,GSK3A,ITGAL,KRT18, LAMB2,NID2,NPM1,PFN1,PMVK,RAI14,SDC2,SERPINC1, SERPINF2,SFRP4,SLC31A2,SPOCK3,TAF1,VAPB,VAPB,VPS62,ZNF410
	CM23	**Yellow**	0*	**0.994**	0*	0.93	Dysfunctional	Positive regulation of cell proliferation, immune system development	ACP1,CENPC1,FKBP7,GPS2,HBXAP,LCK,LTBR,NCL,PIK3R1,SDCCAG8, SLC22A7,SRC,TAF11,UBE1C

Oncogenic modules are formed upon viral infection and preserved in HCC, dysfunctional modules are preserved in normal status but disrupted in HCC. Bold font corresponds to significant disruption (score > 0.95), and asterisk corresponds to significant preservation (score < 0.05). If a module has both significant disruption and preservation in the same stage of progression, only disruption is considered.

Table 2 Top enriched functional annotation clustering of HBV and HCV human protein targets

Virus	Cluster	Functional annotation	*P*-value	FDR
HBV	Cluster 1	hsa05200:Pathways in cancer	4.33E−27	4.98E−25
		hsa04115:p53 signaling pathway	6.91E−12	7.94E−10
		hsa04110:Cell cycle	5.09E−08	5.86E−06
	Cluster 2	hsa04920:Adipocytokine signaling pathway	1.12E−05	1.29E−03
		P00036:Interleukin signaling pathway	1.29E−05	7.35E−04
	Cluster 3	P00006:Apoptosis signaling pathway	6.19E−18	3.53E−16
		hsa04210:Apoptosis	2.01E−12	2.31E−10
HCV	Cluster 1	hsa04510:Focal adhesion	2.32E−08	2.74E−06
		REACT_13552:Integrin cell surface interactions	2.86E−07	1.52E−05
	Cluster 2	hsa04520:Adherens junction	1.64E−05	1.93E−03
	Cluster 3	hsa05200:Pathways in cancer	2.71E−08	3.19E−06
		P04398:p53 pathway feedback loops	3.99E−04	3.14E−02

exact test revealed several pairs of oncogenic and dysfunctional modules with a significant overlap ($P < 0.05$; Figure 6). Especially, we noticed that 3 HBV oncogenic modules (BM2, BM15, BM23) and 2 HCV oncogenic modules (CM18, CM22) have significant overlap with each other, e.g. BM2 with CM18 (Figure 7A and B), BM15 with CM18, and BM23 with CM22 (Figure 7C and D), representing the subsets of genes consistently coexpressed upon viral infection in both HCV- and HBV-infected status. We reasoned that it is these common subsets of genes that lead to carcinogenesis, and such genes can only be extracted by comparing the overlap between HBV and HCV modules. The documented HCC genes curated from literature (Wu et al., 2010) are marked as red in Figure 7. Although shared by overlapping modules, they occupy different network positions (intramodular connectivity, corresponding to the node size) and have different interacting partners (corresponding to their strongest first neighbors).

Comparison of functional enrichment. Secondly, common pathways of biological process were found in both HBV and HCV modules, which were associated with a wide range of functions that can be grouped into several categories: regulation of apoptosis, immune response, inflammation, cell cycle, cell migration, intracellular transport, signal transduction, and nitrogen compound catabolic process (Table 2). They represent general dysfunctional processes that are related to carcinogenesis, regardless of viral types. Distinct functional annotation clusters were also identified, which suggests the differences between HBV and HCV. A detailed functional enrichment of GO annotations in these modules is provided in the Supplementary Tables S3 (HBV modules) and S4 (HCV modules), and all GO terms mentioned in this section are highlighted in yellow background to facilitate search.

We are most interested in inflammation-related oncogenic modules, because they indicate the oncogenic processes that are directly activated by virus (these modules are recapitulated in HCC but not in normal liver tissues). The most contrasting distinction is that positive regulation of apoptosis (BM2, HBV, blue, 3.89E−6), programmed cell death (BM2, HBV, blue, 4.62E−5)

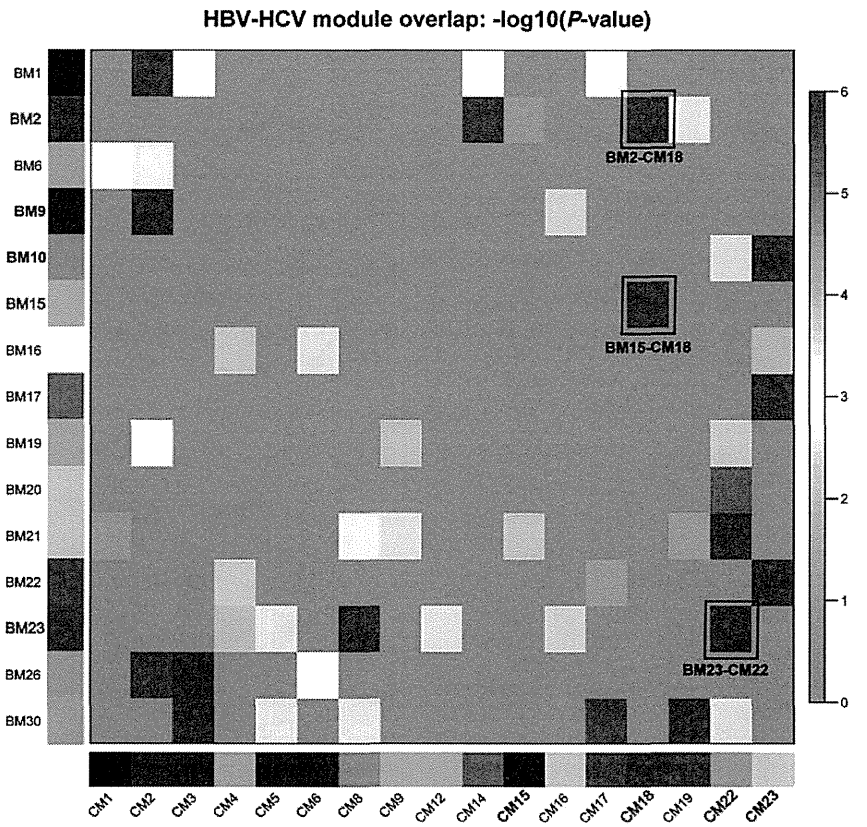


Figure 6 Overlap in gene compositions between HBV and HCV modules. Significance of pairwise module–module overlap was based on Fisher's exact test *P*-values. All HBV oncogenic modules (BM2, BM15, BM23) and HCV oncogenic modules (CM18, CM22) have significant overlap with each other, e.g. BM2 with CM18, BM15 with CM18, BM23 with CM22, representing smaller subsets of genes within modules that are consistently coexpressed in both HBV- and HCV-liver tissues. However, it is the different network properties and combinations of these subsets of genes that lead to the distinct functional annotations enriched in the corresponding HBV and HCV modules.

and cell death (BM2, HBV, blue, 5.05E−6) were top enriched in HBV infection-related modules whereas negative regulation of apoptosis (CM22, HCV, turquoise, 4.0E−6), programmed cell death (CM22, HCV, turquoise, 5.73E−6) and cell death (CM22, HCV, turquoise, 6.12E−6) were top enriched in HCV infection-related modules. The HCV oncogenic module was also top enriched in positive regulation of transcription (CM22, HCV, turquoise, 1.69E−6). This is in concordance with previous research findings that anti-apoptosis is predominant in HCV while pro-apoptosis is predominant in HBV, and that transcription regulation is activated in HCV (Honda et al., 2006). As is summarized previously (Bouchard and Navas-Martin, 2011), one of the mechanisms for HBV-induced HCC is the endless cycle of destruction of HBV-infected hepatocytes by immune cells and concomitant liver regeneration, during which a mutagenic environment is generated. In HCV-induced HCC, however, chronic inflammation that changes the microenvironment but does not lead to immediate death of infected hepatocytes plays the leading role. In fact, HCV core protein targets several tumor suppressor proteins (such as P53, P73, and pRb; Zhang and Horvath, 2005), and HCV nonstructural NS5A protein can block the cell death activity while promoting cell survival pathways by interacting with various cellular regulators (Lan et al., 2002; Chung et al., 2003).

We observed that intracellular transport (BM23, HBV, red, 5.37E−4), intracellular protein transmembrane transport (BM23, HBV, red, 9.02E−3), and transmembrane receptor protein tyrosine kinase signaling pathway (BM23, HBV, red, 1.20E−2) were top enriched in a HBV oncogenic module. Since cell surface receptor and intracellular signaling factors define the host range of HBV (Seeger et al., 2007), these processes can be related to the entry of uncoated HBV into hepatocytes. Interestingly, nucleocytoplasmic transport (BM23, HBV, red, 0.044) and nuclear transport (BM23, HBV, red, 0.047) are uniquely, although marginally, enriched in the HBV oncogenic module, which is consistent with the fact that HBV is able to transport its DNA genome into the nucleus (Rabe et al., 2009). For HCV, endosome to lysosome transport (CM15, midnightblue, 3.46E−3) and endosome transport (CM15, midnightblue, 5.95E−3) were top enriched in a dysfunctional module. Since endosome and lysosome are compartments of the endocytic membrane transport pathway, this is consistent with our existing knowledge that the whole body of HCV enters hepatocytes via endocytosis (Ashfaq et al., 2011). Compared with HCV, intracellular transport can play more important roles in carcinogenesis in HBV.