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Mixed cryoglobulinemia decelerates hepatocellular carcinoma development in hepatitis C patients with SVR by downregulating regulatory B cells: a 12-year prospective cohort study

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ABSTRACT

How mixed cryoglobulinemia (MC) affects cancer risk in chronic hepatitis C patients with sustained virologic response (SVR) remains unclear. In a 12-year prospective study, post-SVR MC was assessed every 3–6 months. Among the 891 SVR patients, 265 (29.7%) had baseline (24 weeks after completing anti-HCV therapy) MC, and the 12-year cancer cumulative incidence was 19.7%. Among the 73 patients who developed cancer, 37 (50.7%) had hepatocellular carcinoma (HCC), with the following associated baseline variables: for cancer, male sex, age and alanine aminotransferase (ALT) levels; for HCC, male sex, age, and cirrhosis; and for non-HCC cancer, rheumatoid factor levels. Among patients with post-SVR HCC, the mean time to HCC was longer in those with than in those without baseline MC (1545.4 ± 276.5 vs. 856.9 ± 115.2 days, $p = 0.014$). Patients with baseline MC had decreased circulating interleukin-10 (IL-10)-positive B cell (CD19+IL-10+cells/CD19+cells) (31.24 ± 16.14 vs. 40.08 ± 15.42%, $p = 0.031$), regulatory B cell (Breg) (CD19+CD24hi CD27+cells/CD19+cells) (10.45 ± 7.10 vs. 15.76 ± 9.14%, $p = 0.035$), IL-10-positive Breg (CD19+CD24hiCD27+IL-10+cells/CD19+cells) (5.06 ± 4.68 vs. 8.83 ± 5.46%, $p = 0.015$) and HCC-infiltrating Breg (18.6 ± 10 vs. 33.51 ± 6.8%, $p = 0.022$) ratios but comparable circulating and HCC-infiltrating regulatory T cell ratios relative to patients without baseline MC. In conclusion, old male SVR patients with elevated ALT levels or cirrhosis require intensive monitoring for cancer development, especially HCC. Tailored HCC follow-up is needed for SVR patients according to their baseline MC, which might downregulate Bregs to decelerate HCC development for almost 2 years.

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Introduction

Hepatitis C virus (HCV), a human pathogen responsible for acute and chronic liver disease that chronically infects an estimated 71.1 million individuals worldwide,[1] is classified into 8 genotypes.[2] HCV infection leads to hepatic complications such as hepatic steatosis, cirrhosis and hepatocellular carcinoma (HCC), as well as many extrahepatic complications, including mixed cryoglobulinemia,[3] hypolipidemia, diabetes mellitus and cardiovascular events.[4,5] Mixed cryoglobulinemia is the most common HCV-associated extrahepatic complication, such that up to 90% of patients with mixed cryoglobulinemia present with HCV infection,[6] and approximately 60% of chronic hepatitis C (CHC) patients have mixed cryoglobulinemia.[7] Although anti-HCV therapy has beneficial effects on curing HCV-associated mixed cryoglobulinemia, failure to cure mixed cryoglobulinemia or relapse of mixed cryoglobulinemia is not uncommon in CHC patients despite a sustained virologic response (SVR) following anti-HCV therapy.[7,8] Mixed cryoglobulinemia is thus a major issue for CHC patients regardless of SVR.

High plasminogen activator inhibitor 1 (PAI-1) expression is predictive of poor long-term prognosis in HCC,[9] and PAI-1 distorts autocrine transforming growth factor-beta signals to accelerate the malignant potential of HCC.[10] Moreover, activated hepatic stellate cells exert immunosuppressive effects in HCC by producing complement component 3 (C3),[11] and the C3a fragment is a biomarker for HCV-related HCC.[12] Interestingly, our previous CHC studies demonstrated the involvement of the PAI-1 pathway in the development of mixed cryoglobulinemia,[13] the interactive impacts of HCV infection and mixed cryoglobulinemia on complement levels[14] and the increasing trends of serum PAI-1[15] and C3[14] levels in SVR patients. All of the above findings suggest that mixed cryoglobulinemia might be crucial for HCC development in CHC patients even after SVR. A 15-year cohort study of 950 untreated CHC patients revealed a lower risk of HCC in patients with baseline cryoglobulinemic vasculitis than in their counterparts.[16] In contrast, in another study of 380 CHC patients treated with direct-acting antiviral agents (DAAs), pretherapy mixed cryoglobulinemia was positively associated with HCC development.[17] Considering the inconclusive effect

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of pretherapy mixed cryoglobulinemia on HCC risk in CHC patients, the manner in which post-SVR mixed cryoglobulinemia affects HCC risk in SVR patients is markedly unclear. Non-HCC cancers, including B-cell non-Hodgkin lymphoma,[18] papillary thyroid cancer,[19,20] and lung adenocarcinoma,[21] have been linked with HCV-associated mixed cryoglobulinemia, but how post-SVR mixed cryoglobulinemia impacts the risk of developing cancers other than HCC also remains unknown.

Accordingly, we aimed to elucidate the effect of post-SVR mixed cryoglobulinemia on the risk of cancer emergence in SVR patients in Taiwan, an Asian country where HCV infection is endemic. This 12-year prospective study evaluated the presence of mixed cryoglobulinemia and cancer development every 3 to 6 months in SVR patients during post-SVR follow-up. The associated immunological basis was also investigated.

Materials and methods

Patients

CHC patients aged 18 years and older who had achieved SVR following a course of anti-HCV therapy with pegylated interferon-α-2b and ribavirin for either 24 or 48 weeks on the basis of the viral genotype[4,5,7,8] or with various combinations of DAAs[8] (Supplementary Table S1) based on the reimbursement policy of the Bureau of National Health Insurance were consecutively recruited. CHC was defined as the presence of a detectable level of serum HCV RNA by polymerase chain reaction for ≥24 weeks, and SVR was defined as an undetectable level of HCV RNA at 24 and 12 weeks after the completion of interferon (IFN)-based therapy or DAA-based therapy, respectively. Mixed cryoglobulinemia was defined as the presence of mixed cryoglobulins in the serum.[22] The diagnosis of mixed cryoglobulinemic syndrome or cryoglobulinemic vasculitis was based on the presence of serum cryoglobulins in ≥2 samples over ≥12-week intervals and ≥2 of 3 positive results in various evaluations, including questionnaires, clinical assessments, and laboratory tests.[7] Subjects with baseline hepatitis B virus or human immunodeficiency virus infection, hemochromatosis, autoimmune liver diseases, extrahepatic autoimmune diseases, cardiovascular and neurologic events, or cancer or who were recipients of solid organ transplants were excluded. In addition, patients who developed cancer within 24 weeks after cessation of anti-HCV therapy were considered to have preexisting cancers and were therefore excluded.

Clinical study

Between July 2010 and July 2022, a total of 891 patients who achieved SVR were recruited from a Taiwan tertiary care center (Figure 1). The observations in this study were conducted prospectively over 12 years (2011–2023). Several baseline factors, including age, sex, smoking, alcohol consumption, body mass index (BMI), the presence of cryoglobulins and liver cirrhosis, the homeostatic model assessment for insulin resistance (HOMA-IR) index, the estimated glomerular filtration rate (eGFR), the Fibrosis-4 (FIB-4) index [Given that FIB-4 > 3.25 indicates likely advanced fibrosis or cirrhosis,[23] we used FIB-4 (3.25) as a categorial factor: >3.25 = 1, ≤3.25 = 0 in multivariate analyses], the levels of alanine aminotransferase (ALT), triglycerides (TG), total cholesterol (TC), rheumatoid factor (RF), immunoglobulin M (IgM), IgG, C3 and C4, and the single-nucleotide polymorphism rs12979860 of interferon-λ3[7] were surveyed 6 months after the completion of anti-HCV therapy. The cryoglobulin levels were measured using the double immunodiffusion method.[24] Biochemical tests were performed at the clinical pathology laboratories of the hospital using routine automated techniques. Incident cancers of the SVR patients were surveyed by assessing the associated symptoms and performing examinations when indicated. The cancers were diagnosed on the basis of the pathologic findings and were confirmed by specialists. Mixed cryoglobulinemia, the aforementioned baseline factors, and emerging cancer were assessed every 3 to 6 months after SVR from December 2011 to December 2023. The TNM (T = primary tumor; N = regional lymph nodes; M = distant metastasis) stages of emergent cancer were recorded.

Flow cytometry

The isolated peripheral blood mononuclear cells (PBMCs) were first resuspended in cell staining buffer (phosphate-buffered saline (PBS) supplemented with 0.5% fetal bovine serum (FBS)) and then subjected to staining with a designated panel of commercial antibodies according to the cell type. To stain the regulatory T cell (Treg) population, anti-CD3-BV510 (clone SK7; BioLegend, CA, USA), anti-CD4-PerCP (clone SK3; BioLegend, CA, USA) and anti-CD45RA-FITC (clone HI100; BD Pharmingen, CA, USA) were used to stain surface markers, and anti-Foxp3-APC (clone PCH101; eBioscience, CA, USA) was used to stain intracellular markers. The recommended volume of each antibody was added to reach a concentration of 1 μg/10^6 cells, and one million cells were used for each test. After staining, the cells were washed and resuspended in cell staining buffer before passing through a flow cytometer (BD FACSCanto II, CA, USA). In brief, CD3+ lymphocytes were gated after gating lymphocytes. Among the gated CD3+ lymphocytes, CD4+ FoxP3+ lymphocytes were chosen as Tregs. Among the Tregs, CD45RA+ and CD45RA- lymphocytes were considered naïve and memory Tregs, respectively. To stain the regulatory B cell (Breg) population,[25] anti-CD19 -FITC (clone: HIB19; BD Pharmingen, CA, USA), anti-CD24 -BV421 (clone: ML5; BD Pharmingen, CA, USA), anti-CD27 -PE (clone: M-T271; BD Pharmingen, CA, USA), anti-CD38-PerCP-Cy5.5 (clone: HIT2; BD Pharmingen, CA, USA) and anti-interleukin 10 (IL-10)-APC (clone: JES3-19F1; BD Pharmingen, CA, USA) were used. To measure intracellular IL-10 levels, PBMCs were stimulated with PMA (50 ng/ml) (Sigma) and ionomycin (500 ng/ml) (Sigma) and with GolgiStop (0.67 μl/ml) for 4 hours and then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, CA, USA) to stain for intracellular markers. In brief, CD19+ lymphocytes were gated after gating lymphocytes. Among the gated CD19+ lymphocytes, CD19+ CD24hi CD27+ lymphocytes were considered Bregs, CD19+ CD24hi CD38hi

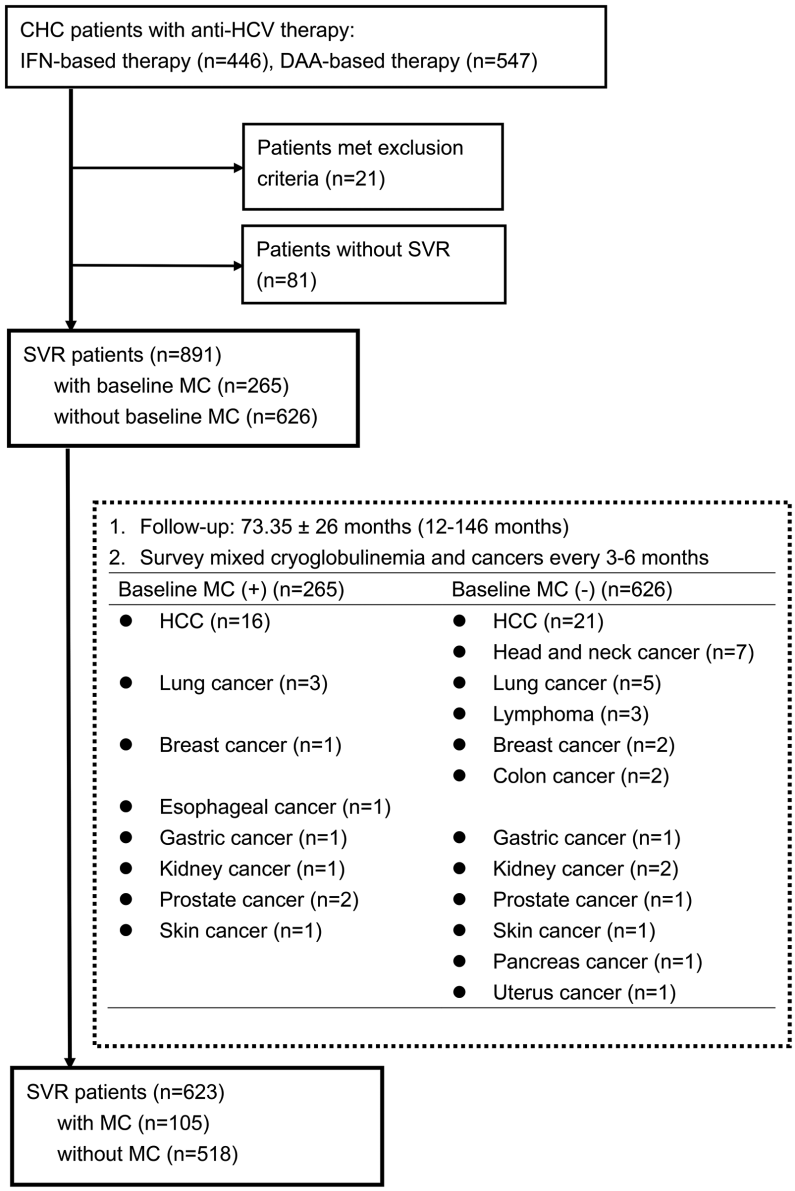

Figure 1. A schematic flow chart of the enrolled patients. CHC: chronic hepatitis C; IFN: interferon; SVR: sustained virological response. MC: mixed cryoglobulinemia.

lymphocytes were considered immature B cells, and CD19+ IL-10+ lymphocytes were considered IL-10+ lymphocytes. The data were analyzed by using FlowJo v10 software.

Immunohistochemistry (IHC) of HCC

Serial sections of paraffinized liver samples from patients who underwent hepatectomy for HCC resection were cut according to the manufacturers' protocols. For liver samples, IHC analysis of B cells (CD20+; R&D Systems, Inc., Minneapolis, MN, USA), Bregs (PAX5+; Abcam Corp., MA, USA),[26] helper T (Th) cells (CD4+; Abcam Corp., MA, USA), cytotoxic T (Tc) cells (CD8+; Abcam Corp., MA, USA) and Tregs (FoxP3+; Abcam Corp., MA, USA) was performed with the corresponding antibodies. Correlations for these proteins were assessed in parallel sections. The intensity of the various markers determined by IHC staining was measured using ImageJ software (http://imagej.nih.gov/ij/, National Institutes of Health, USA).

Statistical analyses

All the statistical analyses were performed using the Statistical Package for Social Science (SPSS package version 21; SPSS, Inc., Chicago, IL, USA), MedCalc (MedCalc version 12.4; MedCalc Software Corp., Maine, USA), Statistical Analysis System (SAS version 9.4; SAS Institute, Inc., Cary, NC, USA) or Prism (GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, CA, USA) software. The Kaplan–Meier method was used to estimate the

cumulative incidence of cancer, HCC or non-HCC cancer. Subdistribution hazards models,[27] modified Cox proportional hazards models that consider competing mortality, were adopted to estimate the adjusted hazard ratios (HRs) for cancer, HCC or non-HCC cancer development and specific independent variables by adjusting for all the independent variables with a p value < 0.157 in the univariate analyses.[28] Receiver operating characteristic (ROC) curve analyses were performed to evaluate the accuracy of the predictors identified through multivariate Cox regression analyses. The Youden index was used to identify the optimum cutoff values of the variables to predict the development of cancer, HCC or non-HCC cancer from the coordinate points of the ROC curves. Generalized estimating equation (GEE) models with a logit link function were adopted to account for longitudinal HCC HR with repeated measurements of all the investigated variables. Among the patients who achieved SVR with HCC development, the normality of the data was checked and confirmed by the Shapiro–Wilk test, and a linear regression model was adopted to compare the difference in time to HCC (the dependent variable) between individuals with and without mixed cryoglobulinemia at baseline (the independent variable).

Results

Baseline characteristics of SVR patients

The baseline variables were measured at 24 weeks after the completion of anti-HCV therapy in SVR patients. Among the 891 SVR patients, 265 (29.7%) had baseline mixed cryoglobulinemia, and 18 (2.0%) had cryoglobulinemic vasculitis. Compared with their counterparts, patients with baseline mixed cryoglobulinemia were more frequently female; had a lower BMI; were more likely to have received interferon-based therapy; had higher rates of cirrhosis; had higher levels of RF, IgG, IgM and FIB-4; and had lower levels of TG and C3 (Table 1).

Emerging cancer in SVR patients

During the 12-year follow-up (range: 12–146 months; mean ± standard deviation: 73.35 ± 26 months), 73 patients developed cancer. The 12-year cumulative incidence of cancer was 19.7%. Among the 73 patients, 37 (50.7%) had HCC. The details of the various cancers are shown in Figure 1. All 7 (9.5%) cases of head and neck cancer and 3 (4.1%) cases of lymphoma occurred in patients without baseline mixed cryoglobulinemia. Multivariate analysis revealed that male sex, baseline age, and baseline ALT levels were positively associated with emerging cancer (Table 2). The age cutoff for predicting cancer development was >58 years (Supplementary Figure S1 A), and the ALT cutoff for predicting cancer development was >13 U/L (Supplementary Figure S1 B).

Emerging HCC in SVR patients

The 12-year cumulative incidences of HCC and non-HCC cancer were 9.8% and 9.9%, respectively. Multivariate analysis revealed that the baseline factors positively associated with HCC development were male sex, age, and cirrhosis [hazard ratio (HR): 3.426] (Table 3); the baseline factor positively associated with emerging non-HCC cancer was baseline RF (Table 4). The cutoff age for predicting HCC development was >53 years (Supplementary Figure S1C), whereas no cutoff of RF could be identified for predicting non-HCC cancer development ($p = 0.654$). Although baseline mixed cryoglobulinemia was not a risk factor for the cumulative incidence of HCC in SVR patients, with a lag period of 689 days (Figure 2), a longer mean time to develop HCC was noted in patients with baseline mixed cryoglobulinemia than in patients without (1545.4 ± 276.5 vs. 856.9 ± 115.2 days, $p = 0.014$; Supplementary Table S2). Among the 16 SVR patients who had baseline mixed cryoglobulinemia and developed HCC, 1 (6.3%) had T1aN0M0 stage I disease, 10 (62.5%) had T1bN0M0 stage IB disease, 2 (12.5%) had T1N0M0 stage I disease, and 3 (18.8%) had T2N0M0 stage II disease. Among the 21 SVR patients

Table 1. Baseline characteristics of the 891 SVR patients.

	All ($n = 891$)	Mixed cryoglobulinemia (+)($n = 265$)	Mixed cryoglobulinemia (-) ($n = 626$)	p values
Male, n (%)	375 (51.1)	97 (25.9)	278 (74.1)	0.016
Age, years	56.32 ± 12.79	57.56 ± 12.69	55.80 ± 12.81	0.087
BMI (kg/m^2)	24.83 ± 3.85	24.27 ± 3.87	25.07 ± 3.82	0.011
ALT (U/L)	22.32 ± 16.31	21.01 ± 13.07	22.88 ± 17.49	0.154
HCV genotype 1, n (%)	415 (56.5)	122 (55.7)	293 (56.9)	0.662
DAA-based therapy, n (%)	409 (55.7)	96 (43.8)	313 (60.8)	<0.001
eGFR (mL/min/1.73 m2)	95.62 ± 32.59	95.49 ± 35.64	95.68 ± 31.21	0.948
TG (mg/dL)	124.35 ± 98.00	109.35 ± 72.94	130.78 ± 106.37	0.007
TC (mg/dL)	185.76 ± 36.16	183.46 ± 37.72	186.74 ± 35.47	0.261
HOMA-IR	2.84 ± 3.16	2.87 ± 3.56	2.83 ± 2.99	0.859
RF(IU/mL)	14.20 ± 21.07	16.13 ± 31.98	13.21 ± 12.12	0.26
IgG (mg/dL)	1498.4 ± 332.9	1563.1 ± 338.7	1466.4 ± 325.8	0.003
IgM (mg/dL)	96.37 ± 53.57	124.68 ± 60.72	82.08 ± 43.09	<0.001
C3 (mg/dL)	107.55 ± 22.08	102.76 ± 17.08	109.99 ± 23.90	0.001
C4 (mg/dL)	21.39 ± 7.93	20.97 ± 8.16	21.60 ± 7.82	0.410
Liver cirrhosis, n (%)	55 (7.5)	17 (7.8)	38 (7.4)	<0.001
FIB-4	2.28 ± 1.76	2.58 ± 2.12	2.13 ± 1.53	0.013
IFNL3–rs12979860 CC genotype, n (%)	477 (87.2)	160 (90.4)	317 (85.7)	0.211

SVR:sustained virological response; BMI: body mass index; ALT: alanine transaminase; DAA: direct-acting antiviral agent; eGFR: estimated glomerular filtration rate; TG: triglycerides; TC: total cholesterol; HOMA-IR: homeostatic model assessment for insulin resistance; RF: rheumatoid factor; IgG: immunoglobulin G; IgM: immunoglobulin M; C3: complement component 3; C4: complement component 4; IFNL3; interferon-λ3.

Table 2. Univariate and multivariate analyses for baseline factors of emerging cancers in SVR patients.

	Univariate		Multivariate	
	95% CI of HR (Estimated HR)	*p* Values	95% CI of HR (Estimated HR)	*p* Values
Sex (male)	1.250 ~ 3.407 (2.064)	0.0046	1.373 ~ 5.160 (2.661)	0.0038
Age (years)	1.031 ~ 1.074 (1.052)	<.0001	1.001 ~ 1.072 (1.036)	0.0436
BMI (kg/m^2)	0.896 ~ 1.024 (0.957)	0.2023		
ALT(U/L)	1.005 ~ 1.026 (1.015)	0.0032	1.006 ~ 1.047 (1.026)	0.0116
HCV genotype	0.638 ~ 0.948 (0.778)	0.0128	0.623 ~ 1.062 (0.813)	0.1283
Therapy (IFN = 1, DAA = 2)	1.542 ~ 4.226 (2.552)	0.0003	0.549 ~ 2.452 (1.160)	0.6978
eGFR (mL/min/1.73 m2)	0.981 ~ 0.996 (0.988)	0.0025	0.984 ~ 1.005 (0.994)	0.2998
TG (mg/dL)	0.996 ~ 1.002 (0.999)	0.628		
TC (mg/dL)	0.986 ~ 1.001 (0.993)	0.0722	0.991 ~ 1.007 (0.999)	0.8044
HOMA-IR	0.913 ~ 1.039 (0.974)	0.4211		
RF (IU/mL)	0.999 ~ 1.026 (1.012)	0.0801	0.992 ~ 1.034 (1.013)	0.242
IgG (mg/dL)	1.000 ~ 1.001 (1.001)	0.013	1.000 ~ 1.002 (1.001)	0.1186
IgM (mg/dL)	0.996 ~ 1.006 (1.001)	0.6815		
C3 (mg/dL)	0.972 ~ 1.001 (0.986)	0.0631	0.968 ~ 1.013 (0.990)	0.3905
C4 (mg/dL)	0.921 ~ 1.012 (0.966)	0.1465	0.948 ~ 1.042 (0.994)	0.8067
Cirrhosis, yes	2.221 ~ 6.204 (3.712)	<.0001	0.844 ~ 5.582 (2.171)	0.1077
FIB-4 (3.25)	2.003 ~ 5.887 (3.434)	<.0001	0.403 ~ 2.301 (0.963)	0.9333
mixed cryoglobulinemia	0.644 ~ 1.677 (1.039)	0.8743		
IFNL3–rs12979860 CC genotype (1,0)	0.505 ~ 2.728 (1.173)	0.7106		

SVR: sustained virological response; CI: confidence interval; HR : hazard ratio; BMI: body mass index; Therapy (1: interferon-based therapy; 2: DAA therapy); ALT: alanine transaminase; eGFR: estimated glomerular filtration rate; TG: triglycerides; TC: total cholesterol; HOMA-IR: homeostatic model assessment insulin resistance; RF: rheumatoid factor; IgG: immunoglobulin G; IgM: immunoglobulin M; C3: complement component 3; C4: complement component 4; MC: mixed cryoglobulinemia; IFNL3: interferon-λ3 (1: CC genotype; 0: non-CC genotype).

Table 3. Univariate and multivariate analyses for baseline factors of emerging HCC in SVR patients.

	Univariate		Multivariate	
	95% CI of HR (Estimated HR)	*p* Values	95% CI of HR (Estimated HR)	*p* Values
Sex (male)	1.730 ~ 8.918 (3.928)	0.0011	1.351 ~ 7.353 (3.152)	0.0079
Age (years)	1.053 ~ 1.108 (1.080)	<.0001	1.033 ~ 1.120 (1.076)	0.0004
BMI (kg/m^2)	0.885 ~ 1.061 (0.969)	0.4998		
ALT(U/L)	1.007 ~ 1.030 (1.019)	0.0012	0.996 ~ 1.025 (1.010)	0.1666
HCV genotype	0.523 ~ 1.003 (0.724)	0.0522	0.499 ~ 1.308 (0.808)	0.3859
Therapy (IFN = 1, DAA = 2)	1.535 ~ 6.183 (3.081)	0.0015	0.581 ~ 3.918 (1.508)	0.3988
eGFR (mL/min/1.73 m2)	0.976 ~ 0.994 (0.985)	0.0008	0.984 ~ 1.010 (0.997)	0.6748
TG (mg/dL)	0.995 ~ 1.003 (0.999)	0.7103		
TC (mg/dL)	0.980 ~ 1.000 (0.990)	0.0468	0.984 ~ 1.007 (0.995)	0.4187
HOMA-IR	0.941 ~ 1.059 (0.998)	0.9594		
RF (IU/mL)	0.983 ~ 1.023 (1.003)	0.7759		
IgG (mg/dL)	1.000 ~ 1.002 (1.001)	0.0938	0.999 ~ 1.001 (1.000)	0.57
IgM (mg/dL)	0.998 ~ 1.008 (1.003)	0.2048		
C3 (mg/dL)	0.967 ~ 1.010 (0.989)	0.3009		
C4 (mg/dL)	0.898 ~ 1.036 (0.965)	0.3247		
Cirrhosis, yes	2.642 ~ 9.960 (5.130)	<.0001	1.106 ~ 10.611 (3.426)	0.0328
FIB-4 (3.25)	2.376 ~ 9.700 (4.801)	<.0001	0.317 ~ 2.676 (0.921)	0.8804
mixed cryoglobulinemia	0.802 ~ 2.838 (1.508)	0.2023		
IFNL3–rs12979860 CC genotype (1,0)	0.323 ~ 2.600 (0.917)	0.8701		

HCC: hepatocellular carcinoma; SVR: sustained virological response; CI: confidence interval; HR : hazard ratio; BMI: body mass index; Therapy (1: interferon-based therapy; 2: DAA therapy); ALT: alanine transaminase; eGFR: estimated glomerular filtration rate; TG: triglycerides; TC: total cholesterol; HOMA-IR: homeostatic model assessment insulin resistance; RF: rheumatoid factor; IgG: immunoglobulin G; IgM: immunoglobulin M; C3: complement component 3; C4: complement component 4; MC: mixed cryoglobulinemia; IFNL3: interferon-λ3 (1: CC genotype; 0: non-CC genotype).

without baseline mixed cryoglobulinemia and who developed HCC, 2 (9.5%) had T1aN0M0 stage IA disease, 6 (28.6%) had T1bN0M0 stage IB disease, 5 (23.8%) had T1N0M0 stage I disease, 5 (23.8%) had T2N0M0 stage II disease, 2 (9.5%) had T4N0M0 stage IIIB disease, and 1 (4.7%) had T4N0M1 stage IVB disease at the time of diagnosis (Supplementary Table S3). Overall, 18.8% and 38% of the SVR patients with and without baseline mixed cryoglobulinemia, respectively, had HCC that was more advanced than stage II (18.8% vs. 38%, $p = 0.182$). From a longitudinal perspective, GEE analyses revealed that age (HR:1.067, $p = 0.0262$) and cirrhosis (HR:12.139, $p = 0.0084$) were associated with the risk of developing HCC (Supplementary Table S4). Among the 623 SVR patients who were followed up for 12 years, 105 had mixed cryoglobulinemia at the last follow-up (i.e., end mixed cryoglobulinemia). Ultimately, 16.8% (105/623) of the SVR patients had end mixed cryoglobulinemia. However, neither the cumulative incidence of HCC (11.2% vs. 7.5%, $p = 0.538$) nor the mean time to HCC development (829.6 ± 156.6 days vs. 679.4 ± 483.6 days, $p = 0.553$) differed between SVR patients with and without end mixed cryoglobulinemia.

Table 4. Univariate and multivariate analyses for baseline factors of emerging non-HCC cancer in SVR patients.

	Univariate		Multivariate	
	95% CI of HR (Estimated HR)	*p* Values	95% CI of HR (Estimated HR)	*p* Values
Sex (male)	0.578 ~ 2.228 (1.135)	0.7137		
Age (years)	0.994 ~ 1.050 (1.021)	0.1299	0.961 ~ 1.046 (1.003)	0.8998
BMI (kg/m^2)	0.857 ~ 1.041 (0.945)	0.252		
ALT(U/L)	0.993 ~ 1.023 (1.008)	0.3031		
HCV genotype	0.658 ~ 1.056 (0.833)	0.1309	0.670 ~ 1.127 (0.869)	0.2907
Therapy (IFN = 1, DAA = 2)	0.939 ~ 4.030 (1.945)	0.0736	0.213 ~ 2.901 (0.786)	0.7184
eGFR (mL/min/1.73 m2)	0.982 ~ 1.006 (0.993)	0.2868		
TG (mg/dL)	0.995 ~ 1.004 (0.999)	0.7782		
TC (mg/dL)	0.988 ~ 1.008 (0.998)	0.6654		
HOMA-IR	0.778 ~ 1.086 (0.919)	0.3205		
RF (IU/mL)	1.001 ~ 1.036 (1.019)	0.0347	1.003 ~ 1.042 (1.022)	0.0255
IgG (mg/dL)	1.000 ~ 1.002 (1.001)	0.0531	1.000 ~ 1.002 (1.001)	0.1457
IgM (mg/dL)	0.986 ~ 1.009 (0.997)	0.6366		
C3 (mg/dL)	0.967 ~ 1.001 (0.984)	0.0617	0.967 ~ 1.006 (0.986)	0.1773
C4 (mg/dL)	0.917 ~ 1.028 (0.971)	0.3097		
Cirrhosis, yes	0.791 ~ 4.607 (1.909)	0.1503	0.453 ~ 6.245 (1.683)	0.4367
FIB-4 (3.25)	0.723 ~ 4.410 (1.786)	0.2085		
mixed cryoglobulinemia	0.312 ~ 1.417 (0.664)	0.29		
IFNL3–rs12979860 CC genotype (1,0)	0.395 ~ 7.073 (1.670)	0.4859		

Non-HCC: non-hepatocellular carcinoma; SVR: sustained virological response; CI: confidence interval; HR : hazard ratio; BMI: body mass index; Therapy (1: interferon-based therapy; 2: DAA therapy); ALT: alanine transaminase; eGFR: estimated glomerular filtration rate; TG: triglycerides; TC: total cholesterol; HOMA-IR: homeostatic model assessment insulin resistance; RF: rheumatoid factor; IgG: immunoglobulin G; IgM: immunoglobulin M; C3: complement component 3; C4: complement component 4; MC: mixed cryoglobulinemia; IFNL3: interferon-λ3 (1: CC genotype; 0: non-CC genotype).

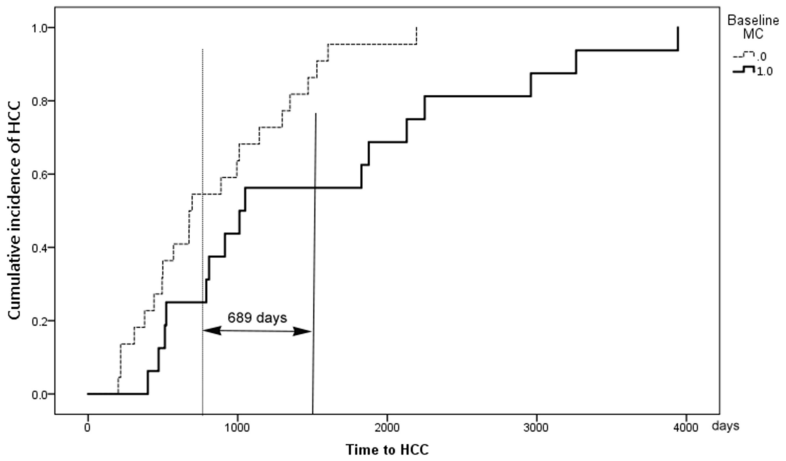

Figure 2. Cumulative incidence of hepatocellular carcinoma (HCC) in SVR patients with (solid line) and without baseline mixed cryoglobulinemia (MC). The lag period to HCC development between patients with and without baseline MC was 689 days (doubled-headed arrow).

Decreased percentages of circulating IL-10-positive B cells, bregs, and IL-10-positive bregs in SVR patients with baseline mixed cryoglobulinemia

Flow cytometry revealed that among SVR patients without any cancer, among the total B cell (CD19+ cell) population, there were lower percentages of IL10-positive B cells (CD19+ IL-10+ cells) (31.24 ± 16.14 vs. 40.08 ± 15.42%, $p = 0.031$) (Figure 3a–d), Bregs (CD19+ CD24hi CD27+ cells) (10.45 ± 7.10 vs. 15.76 ± 9.14%, $p = 0.035$) (Figure 3e,f,g) and IL-10-positive Bregs (5.06 ± 4.68 vs. 8.83 ± 5.46%, $p = 0.015$) (Figure 3h) in SVR patients with baseline mixed cryoglobulinemia than in those without. The percentages of B cells (CD19+ cells) (among lymphocytes) (3.18 ± 1.19 vs. 3.43 ± 1.52%, $p = 0.741$), immature B cells (CD19+ CD24hi CD38hi cells) (among CD19+ cells) (6.86 ± 8.84 vs. 2.93 ± 1.69%, $p = 0.33$), Tregs (CD3+ CD4+ FoxP3+ cells) (among CD3+ CD 4+ cells) (3.59 ± 2.07 vs. 3.54 ± 1.89%, $p = 0.943$) (Figure 3i,j,k), naïve Tregs (CD4+ Foxp3+ CD45RA+ cells) (among Tregs) (5.62 ± 4.85 vs. 6.64 ± 5.78%, $p = 0.591$) and memory Tregs (CD4+Foxp3+CD45RA- cells) (among Tregs) (94.38 ± 4.85 vs. 95.35 ± 5.78%, $p = 0.588$) were similar between SVR patients with and without baseline mixed cryoglobulinemia. However, differences in the percentage of Bregs (10.51 ± 9.7 vs. 6.90 ± 3.58%, $p = 0.425$) and the percentage of IL-10-B cells (28.79 ± 17.97 vs. 35.37 ± 12.74%, $p = 0.54$) between patients with and without baseline mixed cryoglobulinemia were not observed in SVR patients who developed cancer.

Decreased percentage of HCC-infiltrating bregs in SVR patients with baseline mixed cryoglobulinemia

IHC studies of HCC tissues revealed comparable B cell percentages (24.62 ± 2.7 vs. 42.89 ± 12.06%, $p = 0.116$) (Figure 4a,b), CD8+ cell percentages (42.77 ± 4.08 vs.

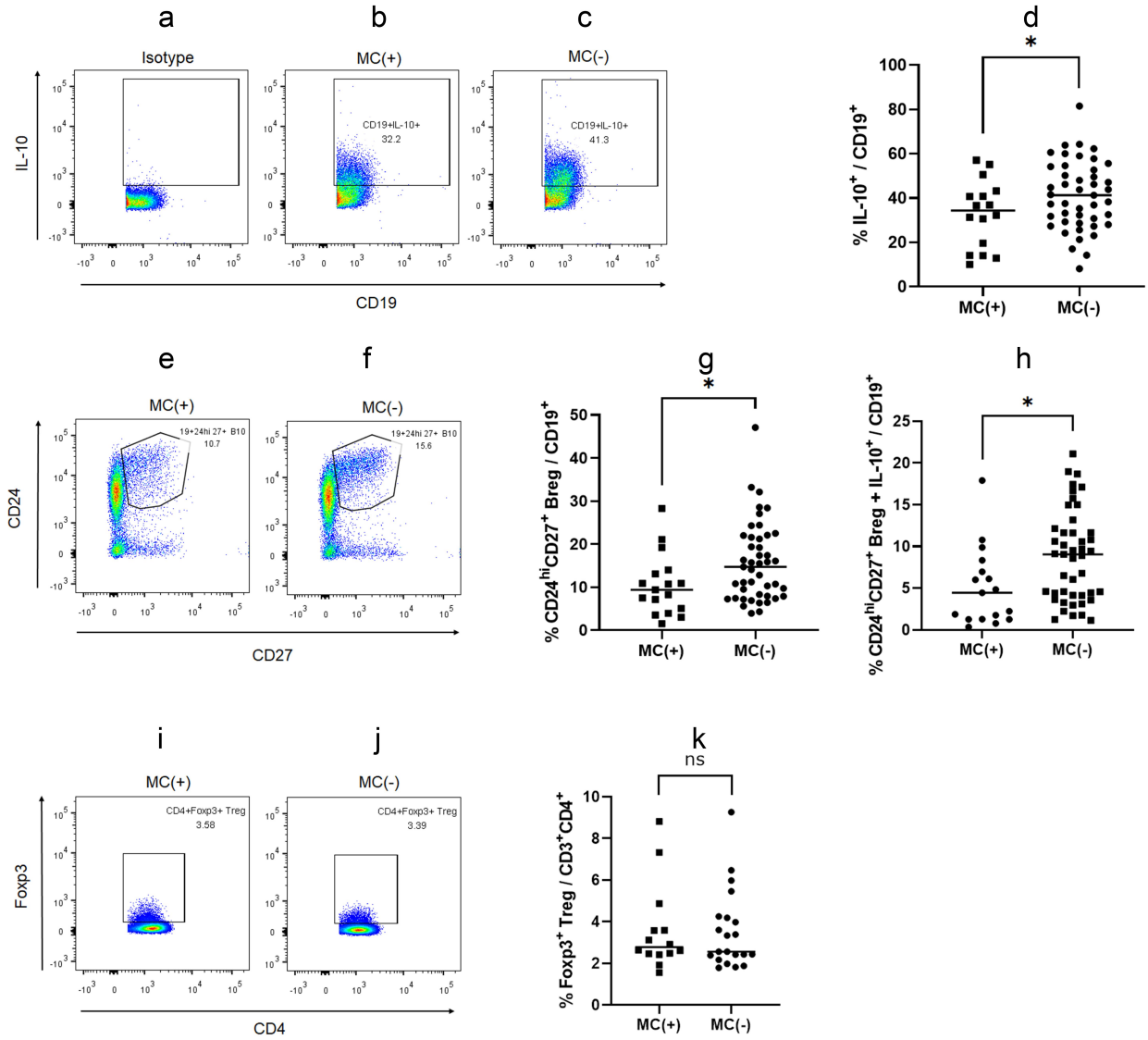

Figure 3. Flow cytometry analyses of peripheral blood mononuclear cells from SVR patients. The immune cell percentages are shown as side scatter values [IL-10 (a, b and c), CD24 (e and f), Foxp3 (I and J)] and forward scatter values [CD19 (a, b and c), CD27 (E and F), and CD4 (I and J)] in a representative patient with baseline mixed cryoglobulinemia (MC (+) (B, E and I) and another patient without baseline MC (MC (-) (c, f and j), as well as scatter plots for IL-10-positive B cells (among CD19 + cells) (D), Bregs (among CD19+ cells) (G), IL-10-positive Bregs (among CD19+ cells) and Tregs (among CD3+ CD4+ cells) (k) of all SVR patients with and without MC. *, significant difference; ns: nonsignificant difference.

35.57 ± 11.14%, $p = 0.447$) (Figure 4e,f), CD4+ cell percentages (56.66 ± 3.56 vs. 43.29 ± 9.01%, $p = 0.126$) (Figure G and H) and Treg percentages (7.37 ± 2.27 vs. 8.63 ± 3.63%, $p = 0.687$) (Figure 4i,j) between patients with and without baseline mixed cryoglobulinemia. However, the percentage of Bregs (18.6 ± 10 vs. 33.51 ± 6.8%, $p = 0.022$) in HCC tissues was lower in patients with than in those without baseline mixed cryoglobulinemia at (Figure 4c,d and Supplementary Figure S2a and b).

Discussion

Our previous study revealed that among patients with spontaneous HCV clearance, the rate of mixed cryoglobulinemia was 37%,[29] which is close to that reported among SVR patients (29.7%) in the present study. Except for the C4 level, at baseline, most of the differences in variables between SVR patients with and without baseline mixed cryoglobulinemia were noted between untreated CHC patients with and without mixed cryoglobulinemia.[7] All the above findings suggest that the results of the current study are reliable and reflect the characteristics of mixed cryoglobulinemia in SVR patients. A decreased C4 level is one of the reliable serological stigmata of HCV-related cryoglobulinemic vasculitis (i.e., mixed cryoglobulinemia syndrome),[30] and lower C4 levels have been detected in untreated CHC patients with mixed cryoglobulinemia than in those without;[7] thus, the comparable C4 level between SVR patients with and without mixed cryoglobulinemia highlights the difference in mixed cryoglobulinemia in CHC patients and in SVR patients and explains why viral clearance attenuates the severity and frequency of HCV-related cryoglobulinemic vasculitis.[7,30]

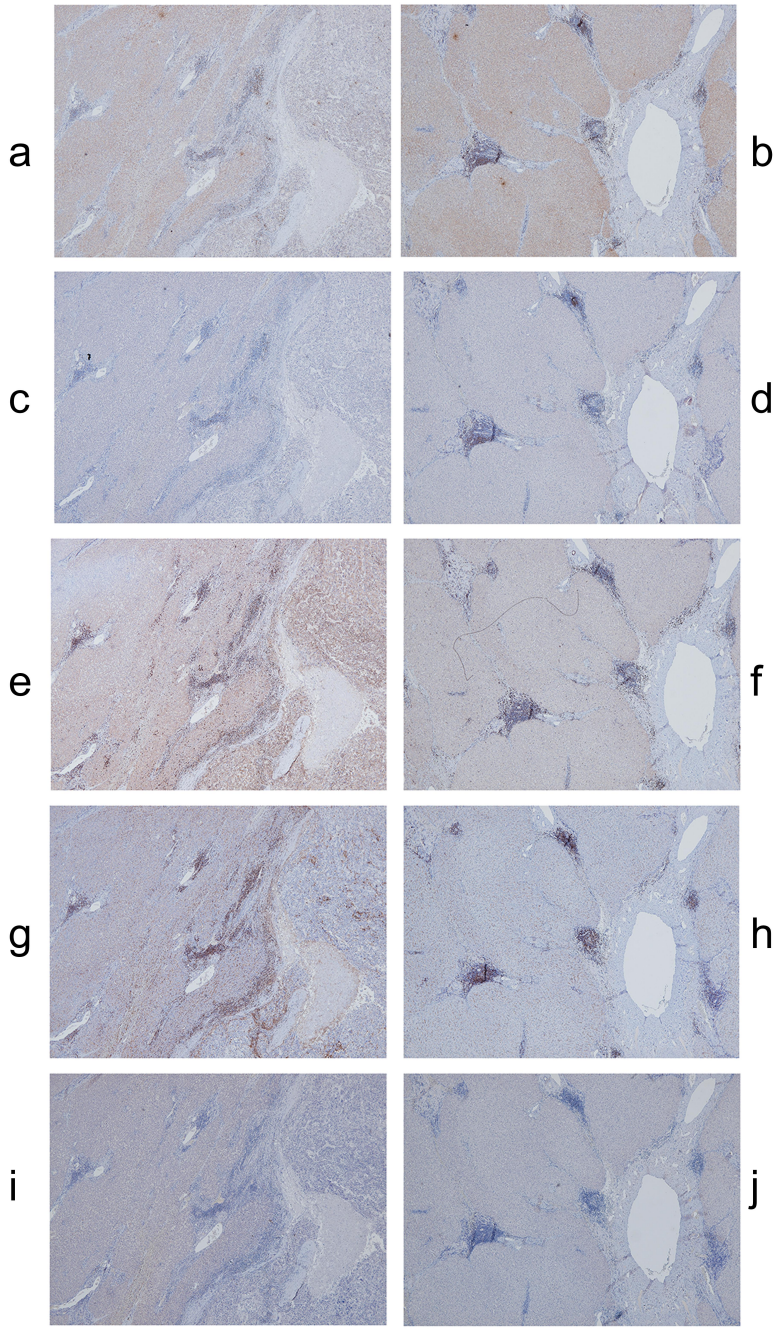

Figure 4. IHC staining of hepatic immune cells in SVR patients who developed HCC with (A, C, E, G and I) or without mixed cryoglobulinemia (B, D, F, H and G). B cells (CD20+ cells) are shown in A and B. Bregs are shown in C and D. CD8+ T cells are shown in E and F. CD4+ T cells are shown in G and H, and Tregs are shown in I and G. All the positive cells exhibit brown staining.

The current study highlighted the 12-year cumulative incidence (19.7%) and the types of cancers that developed in patients who achieved SVR. Consistent with the noted connection between post-SVR mixed cryoglobulinemia and lung cancer,[21] of the 73 SVR patients who developed cancer, 8 patients had lung cancer, and 3 (37.5%) had baseline mixed cryoglobulinemia. However, contrary to previous observations of higher lymphoma[16] and thyroid cancer[19] risks in patients with HCV-associated mixed cryoglobulinemia, none of the patients in the present study developed thyroid cancer, and only 3 cases of lymphoma occurred in patients who did not have mixed cryoglobulinemia. The risk of HCV-associated lymphoma may persist in SVR patients with cryoglobulinemic vasculitis but be attenuated in those with mixed cryoglobulinemia.[16] Compared with mixed cryoglobulinemia, cryoglobulinemic vasculitis is more likely to yield pathogenically transformed B cell clones that develop B cell lymphomas,[31] but the impact of cryoglobulinemic vasculitis on lymphoma might be dampened in the current study since only 4 patients had baseline cryoglobulinemic vasculitis. Likewise, the risk of thyroid cancer might be evident only in patients with cryoglobulinemic vasculitis but not in those with mixed cryoglobulinemia; the associated risk is accentuated by autoimmune thyroiditis,[32] which reproduces the same

multistep process of HCV lymphotropism from mixed cryoglobulinemia to overt B cell malignancy[33] as that of developing lymphoma. On the other hand, 0 and 5 SVR patients with and without baseline mixed cryoglobulinemia, respectively, developed head/neck cancer. Whether mixed cryoglobulinemia has any effect on preventing the development of head and neck cancer requires further investigation.

Given that male sex, baseline age (cutoff: >58 years), and ALT levels were associated with the cumulative incidence of cancer, aged male SVR patients with elevated ALT levels are at risk of emerging cancer and require particular attention. The association of ALT levels with cancer development might stem from cancer risk after steatotic liver disease, which is frequently seen in SVR patients[5] and potentially leads to elevated ALT levels and cancer development.[34] Specifically, in more than half of the 73 patients, the emerging cancer was HCC. Consistent with the idea that SVR patients with cirrhosis carry a high risk of HCC for years,[35] the HR of baseline cirrhosis for HCC was 3.426, and the GEE analyses confirmed that cirrhosis was an independent risk factor for the development of HCC longitudinally. Although no significant differences in the incidence of HCC ≥ stage II were noted between SVR patients with and without baseline mixed cryoglobulinemia, notably, baseline mixed cryoglobulinemia seemed to delay HCC emergence for almost 2 years in SVR patients, as was evident by the mean lag period of 689 days to HCC development noted for HCC patients with baseline mixed cryoglobulinemia. Our previous study revealed that the presence of HCV-associated mixed cryoglobulinemia, even after SVR, likely signifies an immune reaction for suppressing HCV,[13] but the main players involved in this immune reaction remain unidentified. Although Tregs are critical for antagonizing anticancer immune responses and are detrimental to tumor progression,[36] we did not observe any differences in the percentages of circulating or HCC-infiltrating Tregs between SVR patients with and without mixed cryoglobulinemia. On the other hand, mixed cryoglobulinemia indicates B cell expansion[6,7] and may lead to the transformation of B cell clones.[31] Altered B cell subsets are associated with the development and prognosis of HCC.[37] For example, HCC-infiltrating Bregs are associated with an unfavorable prognosis in patients with HCC,[38] and circulating Bregs attenuate HCC development.[37] Specifically, Breg-mediated immunomodulation involves the temporal release of IL-10,[39] a suppressive cytokine that plays a crucial role in controlling inflammation and modulating adaptive immune responses.[40] Consistently, lower percentages of circulating IL-10-positive B cells, Bregs and IL-10-positive Bregs were detected in SVR patients with than in those without baseline mixed cryoglobulinemia, reflecting a less HCC-prone immunity. Interestingly, once HCC develops, probably due to the complex oncogenic immune cascade,[41] the difference in circulating Breg percentages between patients with and without baseline mixed cryoglobulinemia is abolished. However, in HCC tissues, lower percentages of Bregs were still noted in SVR patients with baseline mixed cryoglobulinemia who developed HCC, which is consistent with the scenario that mixed cryoglobulinemia might first decrease the number of circulating Bregs and then decrease the hepatic infiltration of Bregs to decelerate HCC development in the hepatic microenvironment. In contrast, hypertriglyceridemia may decrease cellular immunity and promote the division of cells, thus increasing an individual's susceptibility to cancer.[42] Moreover, an increase in the C3 concentration starts in the early stages of tumorigenesis,[43] the C3a fragment is a potential biomarker for HCV-related HCC,[12] and the knockdown of C3 suppresses hepatic stellate cell-promoted HCC development.[11] Considering that lower peripheral and hepatic Breg percentages and lower TG and C3 levels were noted in SVR patients with than in those without baseline mixed cryoglobulinemia, SVR patients with baseline mixed cryoglobulinemia may be less likely to develop HCC than their counterparts. Intriguingly, although baseline mixed cryoglobulinemia was associated with delayed HCC emergence, the cumulative incidence of HCC was not adequately decreased in SVR patients with baseline mixed cryoglobulinemia. Sex, age, and cirrhosis are stronger factors for determining the cumulative incidence of HCC than mixed cryoglobulinemia is, based on the results of multivariate survival analyses. Given that SVR patients with baseline mixed cryoglobulinemia were more likely to be female and to have cirrhosis than were those without baseline mixed cryoglobulinemia, the impact of baseline mixed cryoglobulinemia on HCC risk might be masked by the counterbalance between the impacts of sex and cirrhosis. In addition, mixed cryoglobulinemia, a prototype of HCV-driven autoimmune disorders,[6,7,13] is affected by the complex interaction between environmental triggering factors and genetic susceptibility.[13] The deceleration of HCC development in SVR patients with baseline mixed cryoglobulinemia highlights the importance of surveying the triggering factors and genetic susceptibility to mixed cryoglobulinemia in SVR patients, with a focus on Bregs; these are promising approaches for evaluating therapeutic targets for HCC. Furthermore, baseline mixed cryoglobulinemia represents a proxy for delaying the development of HCC. Longer and more thorough follow-up for assessing HCC development is needed in SVR patients with baseline mixed cryoglobulinemia than in their counterparts, particularly for those with cancer risk factors such as male sex, advanced age and cirrhosis.

The mixed cryoglobulinemia status waxed and waned in SVR patients following anti-HCV therapy,[7] and 16.8% of the SVR patients had mixed cryoglobulinemia at the end of the current study (i.e., end cryoglobulinemia). Because no differences in the cumulative incidences or emergence times of HCC were noted between patients with and without end mixed cryoglobulinemia and because the GEE analysis removed the longitudinal association between mixed cryoglobulinemia and HCC development, the impact of post-SVR mixed cryoglobulinemia on HCC development seemed to need long time as only baseline but not end or longitudinal mixed cryoglobulinemia affect the HCC development.

Consistent with the connection of RF with incident cancer in patients with spontaneous HCV clearance,[29] the association of RF with non-HCC cancer among SVR patients suggests a bond between rheumatic diseases and cancer, which might occur via the IL-6 pathway[44] and demands further investigation.

In conclusion, the 12-year cumulative incidences of emerging cancer and HCC in SVR patients were 19.7% and 9.8%, respectively. Special caution is needed when monitoring aged male SVR patients with elevated ALT levels or cirrhosis for the development of cancer, especially HCC. However, elevated RF levels warrant attention for non-HCC cancer. A tailored HCC follow-up protocol for SVR patients based on the development of baseline mixed cryoglobulinemia is needed for surveillance of late-onset HCC with a lag period of approximately 2 years, and decreases in Bregs might account for the mixed cryoglobulinemia-associated decelerated HCC development. An examination of the risk factors for mixed cryoglobulinemia, with a focus on Breg-related immune alterations, may pave the way for identifying anti-HCC targets among SVR patients.

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MLC designed and implemented the study, drafted the manuscript, and critically revised the manuscript for important intellectual content. JSC carried out the statistical analysis and manuscript writing. WTC, YJS, CJK and RNC carried out the data collection and manuscript writing. All the authors read and approved the final manuscript.

Author contributions

CRediT: **Ming-Ling Chang:** Conceptualization, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing; **Jur-Shan Cheng:** Formal analysis; **Wei-Ting Chen:** Data curation; **Yi-Jyun Shen:** Formal analysis; **Chia-Jung Kuo:** Data curation; **Rong-Nan Chien:** Data curation.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the corresponding authors.

Our study did not involve the generation of high-throughput sequencing datasets or other large datasets that are routinely deposited in online repositories (e.g., the GEO database).

Ethics approval and consent to participate

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board (IRB) of Chang Gung Memorial Hospital. Written informed consent was obtained from each patient.

IRB numbers: 101-2289B; 04-7005B; 104-7432A3; 104-9710A3

Abbreviations

B-NHL	B-cell non-Hodgkin lymphoma
BMI	body mass index
Breg	regulatory B cell
C3	complement component 3
CHC	chronic hepatitis C
eGFR	estimated glomerular filtration rate
FBS	fetal bovine serum
GEE	generalized estimating equation
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HOMA-IR	homeostatic model assessment for insulin resistance
HR	hazard ratio
IFN	interferon
IL-10	interleukin-10
IgM	immunoglobulin M
MC	mixed cryoglobulinemia
PAI-1	plasminogen activator inhibitor 1
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
RF	rheumatoid factor
ROC	receiver operating characteristic
SVR	sustained virologic response
TC	total cholesterol
Treg	regulatory T cell
TG	triglycerides

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