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Multilectin-assisted fractionation for improved single-dot tissue glycome profiling in clinical glycoproteomics†

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To survey the glycome shift in cancer, single-dot tissue glycome profiling was improved by incorporating a lectin-assisted fractionation. The differential analysis of tissue specimens from colorectal cancer patients (n = 45) revealed that unfucosylated/α2,6-sialylated glycoproteins significantly increased in patients with poor prognoses. The detailed annotation will be an indispensable supplement for cancer-related glyco-biomarker discovery.

The glycome, the repertoire of glycans produced by individual biological systems (*e.g.*, cells, tissues, and organisms), has been widely reported to modulate numerous physiological and pathological states.[1,2] As important posttranslational modifications of proteins, glycans profoundly affect a wide range of biological processes such as apoptosis,[3] angiogenesis,[4] and microbial recognition.[5] In particular, the importance of the glycome has been highlighted by its role in immune responses to various disease states including cancer, which makes the glycome a promising new source for biomarker discovery.[2,6,7] Currently, mining of the glycome for cancer-associated biomarkers represents a new paradigm for cancer diagnosis and prognosis. However, the inherent diversity, complexity and heterogeneity of glycan structures make glycome analysis particularly challenging.[8]

For decades, capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) have been widely used for glycome analysis. CE is a rapid and reliable analytical tool for determining carbohydrate structures, but it is limited by the need for synthetic standards for every glycan analyzed.[8,9] HPLC is regularly used for glycan separation in most glycome analytical laboratories with excellent reproducibility.[10] Like CE, the utility of HPLC is limited to glycome analysis because of the requirement for chemical or enzymatic liberation of glycans from glycoconjugates. MS has become an indispensable technology in the field of glycomics because of its powerful capacity for determining the structural details of glycans. Recently, new analytical technologies have been developed to optimize the MS-based glycome analysis platform.[11,12] However, the requirement for expensive equipment and professional personnel hinders the broad application of these techniques. Recently, lectin microarrays have emerged as an increasingly important technology for glycome investigation.[13] Compared with the technologies described above, lectin microarrays have been recognized as a simple, rapid, highly sensitive, and high-throughput platform that obtains the glycome profile (both *N*- and *O*-glycans) of diverse complex biological samples without releasing the glycan moieties from glycoconjugates.

Comprehensive elucidation of the glycome from biological samples is a significant challenge because of the complexity and microheterogeneity of glycan structures, which considerably hinder glycans from becoming potential informative biomarkers for understanding both normal biological functions and pathological processes.[14,15] Therefore, effective sample fractionation or enrichment is necessary for more in-depth investigation of glycomes. So far, lectin affinity chromatography (especially multilectin affinity chromatography) has been widely employed by researchers to isolate diverse glycan motifs in the extensive mining of glycomes for cancer biomarker discovery.[16,17] The series of lectins used in the chromatography are usually determined with an emphasis on comprehensiveness. This means a lack of systematic determination based on the differential glycome analysis using clinical specimens (*e.g.*, tissue and serum) reduces the efficiency of biomarker discovery.

Recently, with advances in analytical technologies, tissue glycome investigation is gaining momentum in biomarker discovery.[12,18,19] In fact, compared with serum glycome, tissue glycome is a more direct and authentic reflection of the disease state within the corresponding organ. It is now generally recognized that reliable cancer-specific biomarkers should be produced by the cancer cells themselves and are usually present

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in serum at only low levels, especially at the early stage of cancer.[20] For this reason, it is necessary to develop a novel method to explore tissue glycomes in detail. To date, there have been hardly any related reports focused on the development of a strategy for in-depth tissue glycome analysis. Therefore, we developed an ultrasensitive method for glycan analysis targeting small regions (*i.e.*, single-dot tissue on tissue arrays) on formalin-fixed paraffin-embedded (FFPE) tissue sections by means of lectin microarrays.[21,22] Herein, we report an improved strategy integrating tissue arrays, lectin microarrays, and lectin-assisted fractionation techniques, optimized for in-depth tissue glycome investigation, which would be feasible for elucidation of subtle changes during pathological processes. Furthermore, our strategy provided a new idea for designing a sequential combination of lectins for multilectin affinity chromatography in the early phase of biomarker discovery (Fig. 1).

To prove our concept, we investigated the tissue glycome of colorectal cancer (CRC) with the aim of finding reliable CRC-related glyco-biomarkers for clinical application, *i.e.*, prediction of CRC prognosis. To identify the glycan that specifically predicts CRC prognosis as 10 year survival after surgery, we first compared the glycome profiles of single-dot tissues in CRC patients with good (n = 34) and poor (n = 11) prognoses (Table S1, ESI†) based on FFPE colon tissue arrays and lectin microarrays (First LA in Fig. 1, experimental procedure described in the ESI†). The LecChip microarray (GlycoTechnica, Sapporo, Japan) consists of 45 lectins with a broad range of specificities, covering almost all of the important *N*- and *O*-linked glycans. Their carbohydrate specificities are listed in Table S2 (ESI†). Based on unbiased statistical analysis with all of the normalized data (Table S3, ESI†), we found that the signals of AAL and ABA on the LecChip significantly differentiated the two groups of CRC patients ($p < 0.05$). Specifically, the AAL signal was stronger in patients with good prognosis, and the ABA signal was much weaker in patients

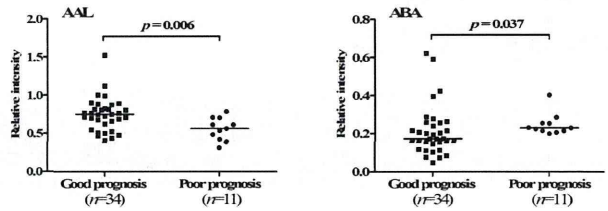

Fig. 2 Selected probe lectins for discrimination of CRC patients with good and poor prognoses.

with good prognosis than in those with poor prognosis (Fig. 2). AAL has specificity for fucosylated glycans,[23] which is known to increase in cancer-associated glycan alterations.[24,25] ABA has specificity for Gal-exposed *O*-linked glycans (*i.e.*, Core1) and GlcNAc-exposed (*i.e.*, agalactosylated) *N*-linked glycans,[26] and is reported to have a potent antineoplastic effect on malignant colon cells.[27,28] Considering the statistical results and the biological significance of AAL and ABA, we selected these two lectins as the probe lectins for further studies.

To further confirm the contribution of selected probe lectins (AAL and ABA) toward CRC prognosis, and for more effective enrichment of potential glyco-biomarker candidates, we combined the lectin microarray-based tissue glycome profiling analysis with lectin-assisted fractionation. For the lectin affinity capturing, 11 patients with good prognosis were selected from the original 34 cases with age (p = 0.92) and sex (p = 0.14) matched to the 11 original patients with poor prognoses. The clinical information including follow-up months for the selected subjects is provided in Table S4 (ESI†).

As shown in Fig. 3A, each Cy3-labeled glycoprotein sample extracted from single-dot tissues was divided into three aliquots (30 μL each) for AAL-affinity capturing, ABA-affinity capturing, and "Input". In our strategy, we collected the supernatants after AAL- and ABA-affinity capturing (*i.e.*, the pass fractions: AAL(−)

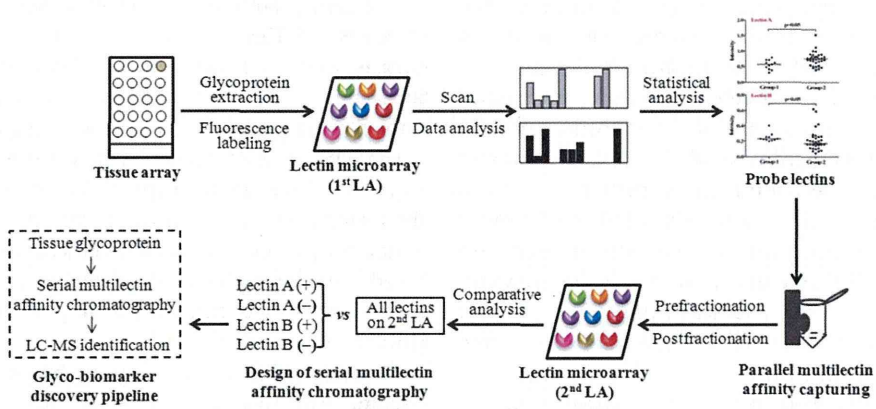

Fig. 1 Schematic diagram for glyco-biomarker discovery based on lectin microarrays and lectin-assisted fractionation. (1) Comparative analysis of tissue glycome profiling between two groups of study subjects based on FFPE tissue arrays and lectin microarrays (1st LA). (2) Statistical analysis for screening the probe lectins to distinguish different groups (*e.g.*, lectins A and B). (3) Fractionation of tissue glycoproteins by affinity capturing using probe lectins in parallel, and then comparative analysis of glycan profiling between the pre- and post-fractionations based on lectin microarrays (2nd LA). (4) Selection of the most accurate biomarker candidates and optimization of the serial multilectin affinity chromatography for glyco-biomarker identification based on LC-MS in further studies.

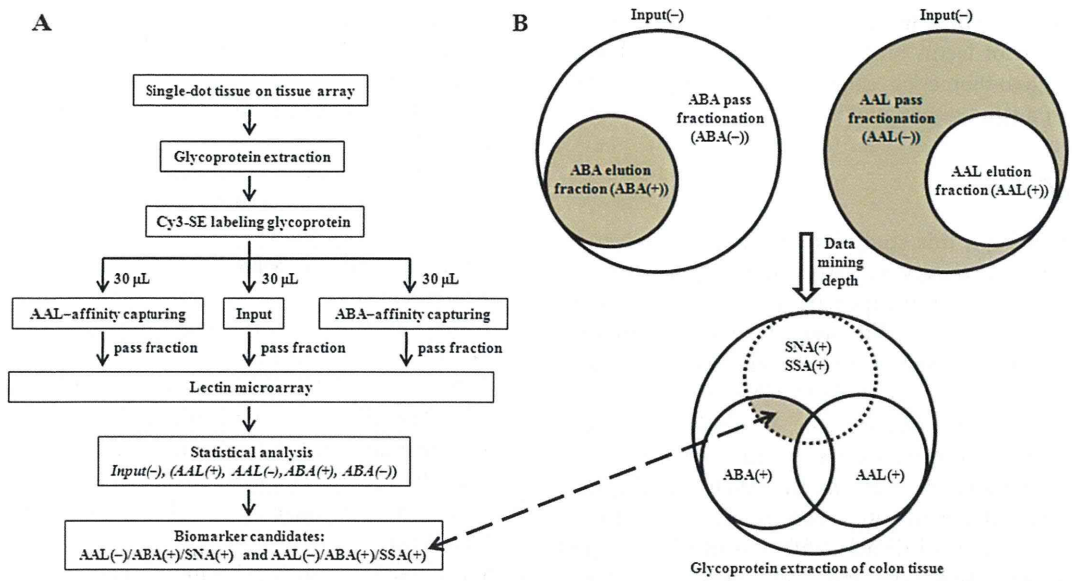

Fig. 3 Mining of potential glyco-biomarkers for CRC prognosis. (A) Scheme for lectin microarray-based tissue glycome profiling analysis with AAL- and ABA-assisted fractionation. (B) A Venn diagram visualizing the results obtained by incorporating the lectin microarrays and lectin-assisted fractionation.

and ABA(−)). Each pass fraction was subjected to lectin microarray analysis (second LA in Fig. 1) instead of the elution fraction in order to minimize the experimental bias. Then, we defined "Input(−) minus AAL(−)" as the AAL captured fraction (*i.e.*, AAL(+)), and "Input(−) minus ABA(−)" as the ABA captured fraction (*i.e.*, ABA(+)). Based on the results of the LecChip analysis with lectin-assisted fractionation (AAL(+), AAL(−), ABA(+), ABA(−)), it was found that the signals of ABA in AAL(−) fractions showed the most significant differences between CRC patients with good and poor prognoses. Furthermore, the signals of α2,6-sialic acid binders such as *Sambucus nigra agglutinin* (SNA) and *Sambucus sieboldiana agglutinin* (SSA) both in AAL(−) and ABA(+) fractions were significantly increased in patients with poor prognosis, whereas both SNA and SSA signals in "Input(−)" showed no significant differences between the two groups of patients (Table 1). It has been reported that the activity of β-galactoside α2,6-sialyltransferase (ST6Gal.I) and SNA reactivity increase in human colon cancer tissues.[29] Taking the specificities of three lectins into account, sialylated mucins, which are well known as potential CRC markers,[30–32] might be rationally enriched in these fractions. This suggested that AAL(−)/ABA(+)/SNA(+) and AAL(−)/ABA(+)/SSA(+) fractions contain a series of glycoproteins, which should be accurate glyco-biomarker candidates for CRC prognosis (Fig. 3B). Considering the potential bias caused by different pathological stages in CRC patients, 5 patients at stage III with good prognoses were selected from 11 patients with age (p = 0.83) and sex (p = 1.0) matched to 5 patients at stage III with poor prognoses for further analysis. The clinical information for the selected subjects is provided in Table S6 (ESI†). The signals of ABA in AAL(−) fractions also showed the largest differences between the two groups, despite the significance becoming weaker (p = 0.058) because of the limited sample size (5 *vs.* 5). Similarly, the signals of SNA and SSA in AAL(−) and ABA(+) fractions also increased in patients with poor prognosis (Table S7, ESI†). These results suggested the potential clinical value of our findings for CRC prognosis while comparing the patient groups at the same pathological stage. All results proved that it is necessary to fractionate glycoproteins for in-depth glycome analysis, and our method was successful in solving the technical problem. We propose an optimized scheme of serial multilectin affinity chromatography for MS-based glycoproteomics, which would contribute towards effectively specifying the target range needed to find novel and clinically promising biomarkers for

Table 1 Significant lectins in AAL and ABA affinity fractionations between CRC patients with good and poor prognoses

	Input(−)			AAL(+)			AAL(−)			ABA(+)			ABA(−)		
Lectin	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p	Good	Poor	p
AAL	23 800	18 000	0.28	23 800	18 000	0.28	0	0	1.00	9200	9000	0.97	14 500	9700	0.12
ABA	6300	9800	0.07	6300	7900	0.49	0	2500	**0.01**	6300	9800	0.07	0	0	1.00
SNA	19 000	20 200	0.22	14 800	13 400	0.87	0	8800	**0.04**	4800	8500	**0.05**	12 100	11 300	0.82
SSA	19 400	20 500	0.21	17 000	14 800	0.87	0	8200	**0.04**	5100	8600	**0.03**	13 500	12 900	0.79

Values are the means of absolute signal intensities. p-values in bold indicate statistical significance by the Mann–Whitney U test. Good prognosis (n = 11), poor prognosis (n = 11).

CRC prognosis (as shown in Fig. S1, ESI†). We will conduct further validation of lectin binding and subsequent identification of the glyco-biomarker candidates in a larger clinical sample pool in a subsequent study.

Conclusions

As a proof of concept, this study confirmed the effectiveness and feasibility of our strategy on lectin microarray-based tissue glycome profiling for clinical applications of glyco-biomarkers. The significant advantages of our strategy include: (1) an ideal combination of an ultrasensitive lectin microarray and a FFPE tissue array with comprehensive clinical information that showed potential advantage in cancer-specific biomarker discovery, (2) simple manipulation and rigorous selection of probe lectins based on statistical analysis to ensure the reliable results, (3) a lectin microarray integrated with lectin-assisted fractionation that can provide more detailed and useful information for logical optimization of serial multilectin affinity chromatography before MS analysis, which would be feasible for glycoproteomics-based biomarker discovery, and (4) a universal platform in the uppermost stream of the pipeline of glyco-biomarker discovery, which can be useful for exploring clinically promising biomarkers in further studies.

Abbreviations

CE	Capillary electrophoresis
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
LA	Lectin microarray
FFPE	Formalin-fixed paraffin-embedded
CRC	Colorectal cancer
AAL	*Aleuria aurantia* lectin
ABA	*Agaricus bisporus* agglutinin
SNA	*Sambucus nigra* agglutinin
SSA	*Sambucus sieboldiana* agglutinin

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HEPATOLOGY

Prediction of liver stiffness hepatocellular carcinoma in chronic hepatitis C patients on interferon-based anti-viral therapy

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chronic hepatitis C, hepatocellular carcinoma, liver stiffness, risk factor.

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Abstract

Background and Aim: The purpose of this study was to evaluate the usefulness of liver stiffness measurement (LSM) for assessing the risk of hepatocellular carcinoma (HCC) in chronic hepatitis C (CHC) patients receiving interferon (IFN) therapy.

Methods: One hundred fifty-one CHC patients who underwent LSM and received IFN therapy were included in the estimation cohort, and 56 were included in the validation study. The cumulative HCC incidences were evaluated using Kaplan–Meier plot analysis and the log-rank test. Multivariate Cox proportional hazard analyses were used to estimate the hazard ratios (HRs) of variables for HCC.

Results: In the estimation cohort, 9 of 151 patients developed HCC during the median follow-up time of 722 days. Multivariate analysis identified three independent risk factors for HCC: LSM ($\geq$ 14.0 kPa, HR 5.58, $P = 0.020$), platelet count ($< 14.1 \times 10^4/\mu L$, HR 5.59, $P = 0.034$), and non-sustained virological response (HR 8.28, $P = 0.049$). The cumulative incidence of HCC development at 3 years was 59.6%, 8.2%, and 0.0% in patients with all three risk factors, one to two risk factors, and none of these risk factors, respectively. The incidence of HCC was significantly different between these groups ($P < 0.001$). In the validation cohort, HCC incidence was also significantly different with respect to these risk factors ($P = 0.037$).

Conclusion: LSM, platelet count, and IFN-therapeutic effect could be used to successfully stratify the risk of HCC in patients receiving IFN therapy and demonstrate the usefulness of LSM before IFN therapy for the management of CHC patients.

Introduction

Persistent hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease leading to the development of HCC, the fifth most common cancer, and the third most common cause of cancer-related death worldwide.[1] HCV is responsible for 27–75% of the HCC cases in Europe and the United States and > 80% of the HCC cases in Japan.[2,3] In fact, HCV-positive patients have a 20-fold higher risk of developing HCC than HCV-negative patients,[4] indicating a significant carcinogenic role for persistent HCV infection. Because of this connection, many chronic hepatitis C (CHC) patients are treated with interferon (IFN)-based antiviral therapy because it not only eradicates HCV but also reduces the rate of HCC development. IFN therapy is most effective at decreasing the risk of developing HCC in patients that achieve a sustained virological response (SVR);[5–7] however, the risk of HCC development persists after IFN therapy even in patients who do achieve SVR.[8] HCC might develop immediately after IFN therapy in some cases, or during long-term IFN therapy in others.[9,10] Because assessing the risk of developing HCC is clinically important in the management of CHC patients, it is necessary to establish predictors for HCC development in patients who receive IFN therapy.

Some factors reported to predict the risk of HCC development after IFN therapy are older age, male gender, and severe fibrosis,[11,12] with advanced fibrosis and cirrhosis significantly correlating with the risk of HCC development.[13] To date, liver biopsy has been the gold standard for assessing the severity of liver fibrosis and cirrhosis,[14] although sampling errors and intraobserver and interobserver variability can lead to understaging.[15,16] In addition, it is difficult to perform liver biopsy for all patients because of its invasiveness and rare but potentially life-threatening complications.[14] As a result, liver stiffness measurement (LSM), a type of transient elastography, has become a reliable alternative for assessing hepatic fibrosis and cirrhosis mainly in patients with CHC.[17,18] LSM is non-invasive, reproducible, can be expressed numerically as continuous values, and has a wide dynamic range in the evaluation of hepatic fibrosis. These advantages over liver biopsy

suggest the clinical usefulness of LSM for predicting HCC development. Here, we evaluated factors that affect the occurrence of HCC in CHC patients receiving IFN therapy, with a special focus on the predictive value of LSM.

Methods

Patients. Between October 2007 and April 2011, a total of 207 consecutive CHC patients who underwent a successful LSM and then received IFN-based antiviral therapy at the Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka, Japan, were retrospectively enrolled in this study. CHC diagnosis was based on serum HCV-RNA positivity. Exclusion criteria were as follows: (i) hepatitis B surface antigen positivity; (ii) other causes of liver disease of mixed etiologies, including autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease; (iii) evidence of hepatocellular carcinoma (HCC) on ultrasonography or computed tomography; (iv) previous history of liver transplantation; and (v) treatment for HCC. This study was approved by the Ethics Committee of Juntendo University Shizuoka Hospital in accordance with the Helsinki Declaration, and all patients provided written informed consent.

Of these 207 patients, 151 underwent ultrasonography-guided percutaneous liver biopsy within a week before treatment initiation. Liver biopsy specimens were embedded in paraffin and stained with hematoxylin-eosin, Azan-Mallory, and reticulin silver impregnation. The specimens were evaluated by an experienced pathologist who was blinded to the patients' clinical data. Histological evaluation was based on the METAVIR criteria.[19] Hepatic fibrosis was defined as follows: F0, no fibrosis; F1, periportal fibrous expansion; F2, portal fibrous widening with bridging fibrosis; F3, bridging fibrosis with lobular distortion; and F4, liver cirrhosis. On the basis of the degree of lymphocyte infiltration and hepatocyte necrosis, inflammation was scored from A0 to A3, with higher scores indicating more severe inflammation. The 151 patients who underwent liver biopsy were enrolled into the estimation group for the identification of risk factors for HCC development, and the remaining 56 patients who did not undergo liver biopsy were enrolled into a group for the validation of these identified risk factors.

All laboratory tests were performed for each patient just before initiation of IFN therapy. Blood cell counts, serum alanine transaminase, gamma-glutamyl transpeptidase, hemoglobin A1c, total bilirubin, albumin, prothrombin time, and alpha-fetoprotein (AFP) were measured using commercially available assays. The HCV genotype was determined using polymerase chain reaction with the HCV Genotype Primer Kit (Institute of Immunology Co., Ltd., Tokyo, Japan) and classified as genotype 1, genotype 2, or other, according to Simmonds' classification system. Serum HCV viral load was determined using quantitative reverse transcription polymerase chain reaction using the COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ, USA).

Treatment protocol. The treatment protocol for CHC patients consisted of 1.5 μg/kg of pegylated IFN-α-2b or 180 μg of pegylated IFN-α-2a once a week, combined with ribavirin at an oral dose of 600–1000 mg/day. Duration of the treatment was 48–72 weeks for those with HCV genotype 1 and a serum HCV viral load > 5 log IU/mL. For all other patients, treatment lasted for 24 weeks. SVR was defined as undetectable serum HCV-RNA at 24 weeks after the end of treatment.

Measurement of liver stiffness. Measurement of liver stiffness by transient elastography was performed using FibroScan (Echosens, Paris, France) within a week before treatment initiation. Technical details of the examination and procedure have been reported previously.[17] Ten validated measurements were made on each patient, and results were expressed in kilopascals (kPa). Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable, and the median value was considered representative of the liver elastic modulus.

Patient follow-up and HCC diagnosis. Serum AFP was measured every month, and ultrasonography or computed tomography were performed at least every 3–6 months for HCC surveillance during and after treatment, with a minimum follow-up duration of 6 months after the initiation of IFN therapy. HCC was diagnosed by histological examination and/or triphasic computerized tomography, in which hyperattenuation in the arterial phase with washout in the late phase is pathognomonic for HCC.[20] The status of patients enrolled in this study was confirmed as of March 2012.

Statistical analyses. All analyses were conducted using IBM SPSS version 19 (IBM SPSS, Chicago, IL, USA), and P values less than 0.05 were considered statistically significant. Continuous variables and categorical variables were summarized as median (range) and percentage, respectively. Mann–Whitney U and chi-square tests were used when appropriate. The strength of the association between LSM and the histological fibrosis stage was estimated using the Spearman's rank correlation coefficient. Cumulative incidences of HCC development were estimated by Kaplan–Meier analysis and compared using the log-rank test. Cox logistic regression analysis was used for multivariate analysis to identify factors that were independently associated with HCC development. The cut-off value of each factor for predicting the development of HCC was determined using receiver operator characteristics analysis.

Results

Patient characteristics. A total of 229 patients received LSM followed by IFN-based antiviral therapy at Juntendo Shizuoka Hospital during the study period. Twenty-two patients (9.6%) were excluded because of LSM failure and/or an invalid LSM. Of the remaining 207 patients, 151 underwent liver biopsy prior to IFN therapy and together formed the risk factor-estimation cohort. The clinical, anthropometric, and laboratory data of the estimation cohort are summarized in Table 1. The 151 patients (83 male and 68 female) had a median age of 62 years (range 22–82 years) and a median LSM of 8.8 kPa (range 2.8–45.7 kPa). There was a significant positive association between LSM and histological fibrosis stage ($r = 0.59$, $P < 0.001$). The prevalence of genotype

Table 1 Baseline characteristics of the estimation cohort

Variables	All	HCC development (+)	HCC development (–)	*P*-value
Number of patients	151	9	142	
Age (years)	62 (22–82)	67 (60–82)	61 (22–80)	0.010†
Male (%)	55	55.6	54.9	1.000‡
BMI (kg/m^2)	23.5 (18.1–36.8)	23.8 (23.3–25.7)	23.4 (18.1–36.8)	0.217†
Habitual drinker (%)	10.6	11.1	10.6	1.000‡
Fibrosis stage (F0–2/F3–4)	115/36	5/4	110/32	0.048‡
Inflammatory grade (A0–1/A2–3)	33/118	0/9	33/109	0.101‡
LSM (kPa)	8.8 (2.8–45.7)	14.8 (9.8–45.7)	8.7 (2.8–34.8)	0.002†
Observation period (days)	722 (189–1378)	688 (189–1217)	733 (190–1378)	0.467†
Genotype 1 (%)	56.3	100	53.5	0.065‡
HCV-RNA (log IU/mL)	6.4 (0.0–7.7)	6.5 (2.9–7.2)	6.3 (0.0–7.7)	0.168†
Albumin (g/dL)	4.1 (3.4–4.8)	4.1 (3.5–4.6)	4.1 (3.4–4.8)	0.390†
ALT (IU/L)	59 (10–410)	75 (27–181)	57 (10–410)	0.467†
Total bilirubin (mg/dL)	0.7 (0.3–1.8)	0.8 (0.5–1.3)	0.7 (0.3–1.8)	0.070†
γGTP (IU/L)	44 (4–517)	75 (31–129)	41 (4–517)	0.120†
Hemoglobin A1c (%)	5.1 (3.7–8.2)	5.1 (3.7–6.1)	5.1 (4.2–8.2)	0.561†
Ferritin (ng/mL)	134 (8–2096)	215 (8–1026)	134 (9–2096)	0.675†
White blood cell count (× 10^3/μL)	4.9 (2.0–10.3)	4.3 (3.0–7.3)	4.9 (2.0–10.3)	0.496†
Hemoglobin (g/dL)	13.8 (8.9–17.5)	13.3 (9.9–17.5)	13.8 (8.9–17.1)	0.376†
Platelet count (× 10^4/μL)	16.3 (5.2–37.0)	9.6 (5.2–19.4)	16.5 (5.8–37.0)	0.004†
Prothrombin time (%)	100 (70–157)	93 (79–120)	102 (70–157)	0.185†
AFP (ng/mL)	6 (1–306)	14 (4–109)	6 (1–306)	0.004†
SVR rate (%)	55	11.1	57.7	0.011‡

Scale data are shown as median (range). *P* values are for comparisons between patients with and without HCC development.
†Mann–Whitney *U* test.
‡Chi-square test.
γGTP, γ-glutamyl transpeptidase; AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LSM, liver stiffness measurement; SVR, sustained virological response.

1 HCV infection was 56.3%. Following IFN-based antiviral therapy, SVR was obtained in 83 of the 151 patients (55%). During the median follow-up period of 722 days (range 189–1378 days), nine patients (6.0%) developed HCC. The cumulative incidence of HCC estimated using the Kaplan–Meier method was 1.3%, 4.5%, and 9.0% at 1, 2, and 3 years, respectively (Fig. 1). Compared with patients who had not developed HCC, HCC patients were of advanced age and had a high LSM, a high fibrosis stage, a low platelet count, and a low SVR rate (Table 1).

Risk analyses. Univariate analysis revealed that age ($P = 0.029$), LSM ($P = 0.005$), platelet count ($P = 0.002$), AFP ($P = 0.003$), and non-SVR ($P = 0.011$) were associated with HCC development (Table 2). Multivariate Cox logistic regression analysis identified three independent risk factors: LSM ≥ 14.0 kPa (hazard ratio [HR] 5.58, 95% confidence interval [CI] 1.32–23.64, $P = 0.02$), non-SVR (HR 8.28, 95% CI 1.01–68.05, $P = 0.049$), and platelet count < 14.1 × 10^4/μL (HR 5.59, 95% CI 1.14–27.53, $P = 0.034$), Table 3. The 1-, 2-, and 3-year cumulative incidence rates of HCC development in patients with LSM < 14.0 kPa were 0.8%, 2.3%, and 4.6%, respectively, whereas those with LSM ≥ 14.0 kPa were 3.2%, 12.0%, and 22.2%, respectively ($P = 0.005$) (Fig. 2a). The cumulative incidence rates of HCC development in patients with SVR were 0.0%, 2.0%, and 2.0%, respectively, whereas those without SVR were 3.0%, 7.4%, and 17.1%, respectively ($P = 0.011$) (Fig. 2b). The cumulative incidence rates of HCC development in patients with a platelet count ≥ 14.1 × 10^4/μL were 0.0%, 0.0%, and 4.2%, respectively, whereas those with a platelet count < 14.1 × 10^4/μL were 4.0%, 13.4%, and 19.1%, respectively ($P = 0.002$) (Fig. 2c).

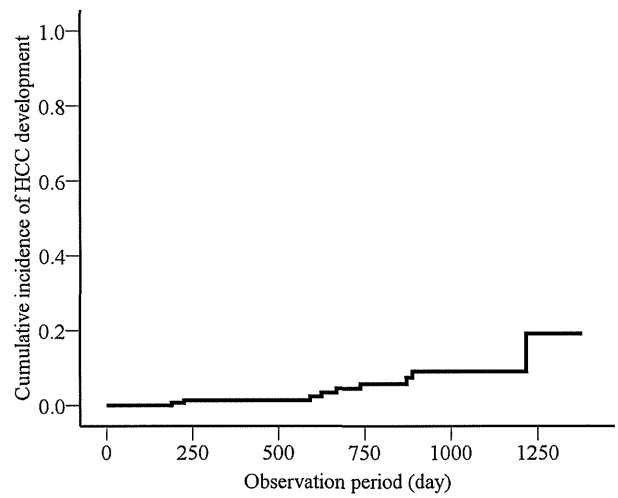

Figure 1 Incidence of hepatocellular carcinoma (HCC) in 151 patients with chronic hepatitis C receiving interferon-based anti-viral therapy estimated using the Kaplan–Meier method.

Table 2 Univariate analysis of factors associated with hepatocellular carcinoma development

Variables	*n*	Cumulative incidence of HCC (%)		*P*-value
		1 year	3 years	
Age (years)				
< 60	63	0.0	0.0	0.029
≥ 60	88	2.3	13.6	
Sex				
Female	68	1.5	12.1	0.910
Male	83	1.2	6.7	
BMI† (kg/m²)				
< 23.8	50	0.0	5.3	0.250
≥ 23.8	42	2.4	6.0	
Habitual drinker				
No	135	0.8	9.6	0.905
Yes	16	6.2	6.2	
Fibrosis stage				
F0–2	115	0.9	6.7	0.228
F3–4	36	2.9	15.0	
LSM (kPa)				
< 14	119	0.8	4.6	0.005
≥ 14	32	3.2	22.2	
ALT (IU/L)				
< 55	71	0.0	4.9	0.123
≥ 55	80	2.5	12.9	
γGTP† (IU/L)				
< 55	83	0.0	5.2	0.057
≥ 55	67	3.0	13.5	
Hemoglobin A1c† (%)				
< 5.5	109	0.9	6.8	0.219
≥ 5.5	25	0.0	18.8	
Ferritin† (ng/mL)				
< 210	74	1.4	10.0	0.175
≥ 210	43	2.3	16.3	
Platelet count (× 10^4/μL)				
≥ 14.1	101	0.0	4.2	0.002
< 14.1	50	4.0	19.1	
AFP† (ng/mL)				
< 10	95	0.0	5.6	0.003
≥ 10	38	4.9	22.3	
SVR				
Yes	83	0.0	2.0	0.011
No	68	3.0	17.1	

†Data not available for all patients.
AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BMI, body mass index; γGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; LSM, liver stiffness measurement; SVR, sustained virological response.

Number of risk factors and HCC development. The number of risk factors varied between patients: 12 patients (7.9%) had all three risk factors, 32 patients (21.2%) had two, 50 patients (33.1%) had one, and 57 patients (37.7%) had none of these risk factors (Fig. 3). Patients without these risk factors did not develop HCC during the study period. In patients with 1 or 2 risk factors, the cumulative incidence rates at 1, 2, and 3 years were 1.2%, 3.1%, and 8.2%, respectively, whereas patients with all three risk

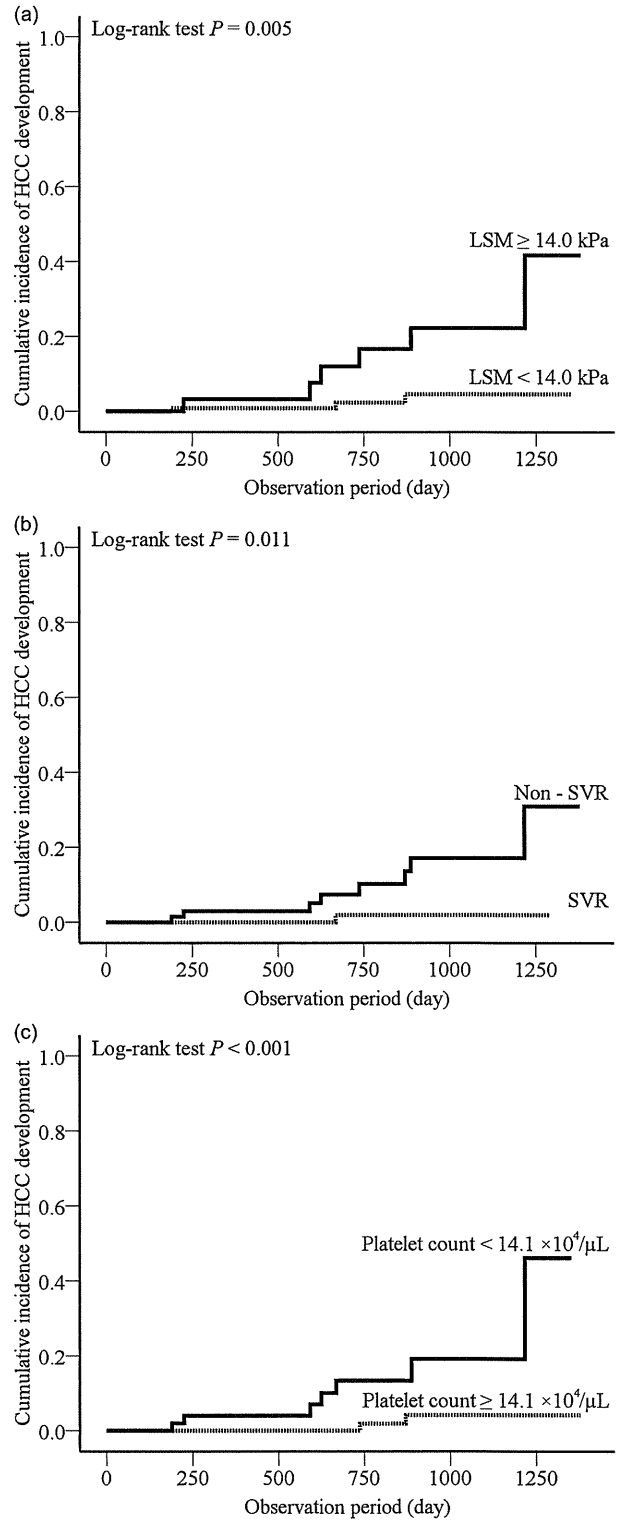

Figure 2 Kaplan–Meier curves comparing the cumulative incidence of hepatocellular carcinoma (HCC) development. Patients were stratified according to liver stiffness measurement (LSM) (a), sustained virological response (SVR) (b), and platelet count (c).