Table 5 Comparison between the patients with HCC and those without HCC in all the 231 patients

	Patients with HCC (n = 48)	Patients without HCC (n = 183)	Comparison between patients with HCC and those without HCC	Multiple regression analysis for factors associated with HCC development	
				Odds ratio (95% confidence interval)	p
Age (yrs)	70.5 ± 7.8	60.9 ± 11.2	p < 0.0001	1.12 (1.07 - 1.17)	p < 0.0001
Gender (male/female)	25/23	78/105	NS	1.83 (0.90 - 3.71)	p = 0.0936
BMI (kg/m^2)	23.1 ± 3.9	22.3 ± 3.4	NS		
Response to IFN treatment (NVR/no past IFN therapy)	17/31	77/106	NS		
PNPLA3 (GG/CC・CG)	34/14	156/27	p = 0.0200	2.62 (1.15 - 5.96)	p = 0.0218
AST (IU/L)	72.1 ± 54.8	53.0 ± 46.4	p = 0.0158		
ALT (IU/L)	60.4 ± 46.4	63.5 ± 82.1	NS		
γ-GTP (IU/L)	50.4 ± 34.9	61.1 ± 85.8	NS		
Albumin (g/dL)	3.5 ± 0.7	4.2 ± 0.5	p < 0.0001		
Total bilirubin (mg/dL)	1.3 ± 0.97	0.9 ± 0.76	p = 0.0010		
Platelet count (x10^4/μL)	9.5 ± 4.2	14.4 ± 5.4	p < 0.0001		
Prothrombin time (%)	83.7 ± 13.90	97.1 ± 18.8	p < 0.0001		
Hyaluronic acid (ng/mL)	473.8 ± 480.9	181.1 ± 267.2	p < 0.0001		
α-fetoprotein (ng/mL)	308.6 ± 1134.9	20.8 ± 113.2	p = 0.0009		
PIVKA-II(mAU/mL)	40.25 ± 43.22	20.56 ± 9.93	p = 0.0030		
HCV genotype (1/2/3)	42/6/0	169/35/2	NS		
HCV RNA (log IU/mL)	6.0 ± 1.1	6.2 ± 1.0	NS		
Velocity of shear wave (m/s)	2.19 ± 0.64	1.57 ± 0.52	p < 0.0001		

HCC, hepatocellular carcinoma; BMI, body mass index; IFN, interferon; NVR, non-virological response; PNPLA3, patatin-like phospholipase domain-containing 3; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; PIVKA-II, protein induced by Vitamin K absence or antagonist-II; NS, not significant.

results of our present study confirmed an association between PNPLA3 and the development of HCC in Japanese patients.

In this study, we demonstrated that a PNPLA3 polymorphism was associated with the progression of fibrosis to cirrhosis. The association was significant by multivariable analysis in the patients without past IFN treatment, while it was only a tendency by analysis in all the 231 patients studied.

Several studies reported that a PNPLA3 polymorphism was associated with fibrosis in patients with CHC (Valenti et al. 2011; Trepo et al. 2011; Valenti et al. 2012; Dunn et al. 2014), while other studies did not find an association between a PNPLA3 polymorphism and fibrosis (Zampino et al. 2013; Rembeck et al. 2012; Miyashita et al. 2012; Nakamura et al. 2013).

These discrepancies reported on the association of PNPLA3 with the development of HCC or fibrosis may be attributed to the difference of the ethnicity, population, and past treatment of the patients studied. In our study, the patients with SVR and relapse of past IFN treatment were excluded, because their LSM results declined and the risk of the development of HCC also was reduced (Arima et al. 2010; Kasahara et al. 1998; Harada et al. 2014). Because the associations of PNPLA3 are not strong for fibrosis (OR = 3.13; 95% CI: 1.50–6.51; P = 0.002) (Trepo et al. 2011) and development of HCC (Trepo et al. 2014), a large number of more homogenous patients should be studied to establish an association by statistical analysis. The present study included 231 patients, and the association between fibrosis and PNPLA3 was shown to be only a tendency by multivariable analysis, while the association was shown by multivariable analysis of the patients without past IFN treatment.

In our present study, we diagnosed cirrhosis on the basis of Vs values rather than by liver biopsy. Some studies reported that an association between Vs values and fibrosis is affected by inflammation (Chen et al. 2012; Yoon et al. 2012), although others denied this association (Bota et al. 2013; Nishikawa et al. 2014; Rizzo et al. 2011). To confirm this association between fibrosis and PNPLA3 in Japanese patients, further studies using liver biopsies are required.

Nishikawa et al. reported that Vs values were negatively correlated with BMI in the patients with fibrosis stage F1 or F2, but not in those with F3 or F4

Table 6 Comparison between the patients with HCC and those without HCC in the 137 patients without past history of IFN treatment

	Patients with HCC (n = 31)	Patients without HCC (n = 106)	Comparison between patients with HCC and those without HCC	Multiple regression analysis for factors associated with HCC development	
				Odds ratio (95% confidence interval)	p
Age (yrs)	70.9±7.1	62.3±11.9	P = 0.0002	1.09 (1.04 - 1.15)	p = 0.0006
Gender (male/female)	16/15	48/58	NS		NS
BMI (kg/m^2)	23.1±4.0	21.9±3.4	NS		
PNPLA3 (GG/CC · CG)	8/23	14/92	P = 0.0928		NS
AST (IU/L)	80.7±64.3	51.4±41.8	P = 0.0032		
ALT (IU/L)	65.5±50.0	63.9±84.1	NS		
γ-GTP (IU/L)	53.5±40.7	60.0±73.6	NS		
Albumin (g/dL)	3.4±0.56	4.2±0.5	p < 0.0001		
Total bilirubin (mg/dL)	1.5±1.1	0.8±0.4	p < 0.0001		
Platelet count (x10^4/μL)	9.2±4.3	14.1±5.5	P = 0.0008		
Prothrombin time (%)	81.5±14.0	96.0±19.7	P= 0.0002		
Hyaluronic acid (ng/mL)	507.0±542.5	202.1±313.7	P = 0.0002		
α-fetoprotein (ng/mL)	475.9±1411.2	28.7±148.1	P = 0.0016		
PIVKA-II(mAU/mL)	47.0±51.0	20.8±10.7	p < 0.0001		
HCV genotype (1/2/3)	27/4/0	82/23/1	NS		
HCV RNA (log IU/mL)	5.9±1.0	6.2±0.9	NS		
Velocity of shear wave (m/s)	2.20±0.70	1.55±0.50	p < 0.0001		

HCC, hepatocellular carcinoma; IFN, interferon; BMI, body mass index; NVR, non-virological response; PNPLA3, patatin-like phospholipase domain-containing 3; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; PIVKA-II, protein induced by Vitamin K absence or antagonist-II; NS, not significant.

(Nishikawa et al. 2014). Bota et al. reported that higher BMI (≥27.7 kg/m^2) were associated with the risk of failed and unreliable measurements of ARFI (Bota et al. 2014). In the present study, BMI was ≥ 27.7 kg/m^2 in 17 patients. Thus we analyzed the 214 patients with BMI < 27.7 kg/m^2. Multivariate analysis showed that older age (OR = 1.06; 95% CI: 1.03–1.09; p = 0.0001), higher BMI (OR = 1.11; 95% CI: 1.00–1.24; p = 0.0576), and PNPLA3 genotype GG (OR = 2.07; 95% CI: 0.94–4.55; p = 0.0712) were factors independently associated with progression to cirrhosis (data not shown). The standard range of BMI is 18.5 – 24.9 kg/m^2. Thus we analyzed 154 patients with BMI of 18.5 – 24.9 kg/m^2. Neither univariate nor multivariate analysis showed the association of PNPLA3 genotype with cirrhosis (data not shown).

The mechanism underlying the association between a PNPLA3 gene polymorphism with the progression of steatosis, fibrosis, and development of HCC has not been determined. It was recently reported that a PNPLA3 I148M variant promotes the synthesis of hepatic lipid because of a gain of function (Kumari et al. 2012). Steatosis maintained by the PNPLA3 genotype I148M may promote the progression of fibrosis and development of HCC (Valenti et al. 2011; Trepo et al. 2011; Valenti et al. 2012).

Conclusions

In this study, we confirmed that the PNPLA3 genotype I148M was associated with the development of HCC in Japanese patients with CHC, and is one of risk factors for cirrhosis in the patients without past history of IFN treatment. Further studies are required to clarify the mechanism underlying this association.

Methods

Patients

Two hundred thirty-one patients with chronic HCV infection consulted with the Department of Liver, Biliary Tract and Pancreas Diseases, Fujita Health University Hospital from May 2010 to October 2012 (Table 1). Of these patients, 137 had no past history of IFN treatment. The other 94 patients had a past history of IFN treatment, for which HCV RNA did not become negative during treatment and their results were considered as NVR. The patients with a past history of IFN treatment and who had achieved a SVR or relapse, which indicated temporary HCV RNA negativity during the treatment, were excluded from the present study because their LSM results declined and the risk of the development of

Table 7 Comparison between the patients with HCC and those without HCC in the 94 patients with NVR of past IFN treatment

	Patients with HCC (n = 17)	Patients without HCC (n = 77)	Comparison between patients with HCC and those without HCC	Multiple regression analysis for factors associated with HCC development	
				Odds ratio (95% confidence interval)	p
Age (yrs)	69.6±9.3	58.9±10.0	P = 0.0001	1.19 (1.08 - 1.32)	p = 0.0007
Gender (male/female)	9/8	47/30	NS		NS
BMI (kg/m^2)	23.2±3.8	22.9±3.3	NS		
PNPLA3 (GG/CC・CG)	6/11	13/64	P = 0.0871	3.95 (1.00 - 15.61)	p = 0.0497
AST (IU/L)	56.35±25.5	55.3±52.3	NS		
ALT (IU/L)	51.1±38.6	63.0±80.0	NS		
γ-GTP (IU/L)	44.8±20.6	63.1±100.8	NS		
Albumin (g/dL)	3.5±1.0	4.3±0.4	p < 0.0001		
Total bilirubin (mg/dL)	0.9±0.5	0.9±1.1	NS		
Platelet count (x10^4/μL)	10.1±4.1	14.9±5.4	P = 0.0008		
Prothrombin time (%)	88.0±14.2	98.4±17.3	P = 0.0295		
Hyaluronic acid (ng/mL)	402.2±316.9	153.1±186.6	P = 0.0002		
α-fetoprotein (ng/mL)	23.2±20.6	9.8±12.1	P = 0.0005		
PIVKA-II(mAU/mL)	28.0±19.4	20.2±8.9	P = 0.0134		
HCV genotype (1/2/3)	16/1/0	63/13/1	NS		
HCV RNA (log IU/mL)	6.2±1.2	6.2±1.1	NS		
Velocity of shear wave (m/s)	2.20±0.70	1.60±0.5	P = 0.0002		

HCC, hepatocellular carcinoma; IFN, interferon; NVR, non-virological response; BMI, body mass index; PNPLA3, patatin-like phospholipase domain-containing 3; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; PIVKA-II, protein induced by Vitamin K absence or antagonist-II; NS, not significant.

HCC also reduced (Arima et al. 2010; Kasahara et al. 1998; Harada et al. 2014).

In addition, patients with hepatitis B virus coinfection, human immunodeficiency virus coinfection, alcoholic liver disease, or autoimmune liver disease were not included in the study. This study was approved by the ethics committee of the Fujita Health University and was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 2008. All patients who participated in this study had provided written informed consent.

PNPLA3 rs738409 genotyping

Genomic DNA was extracted from whole blood samples using QIA amp DNA Mini Kits (Qiagen, Tokyo, Japan), according to the manufacturer's protocol. The rs738409 PNPLA3 SNP was genotyped using TaqMan predesigned SNP genotyping assays (Applied Biosystems, Tokyo, Japan), according to the manufacturer's protocol.

ARFI measurements

Vs measurements by ARFI were made with a Siemens ACUSON S2000 (Siemens Japan Co., Ltd., Tokyo, Japan) as previously reported (Nishikawa et al. 2014). Vs values were expressed in meters/second (m/s), and was considered to be proportional to the square root of tissue elasticity.

Statistical analysis

Results are expressed as means ± standard deviations. Group results were compared using chi-square test or Student's *t*-test, as appropriate. Bonferroni corrections were used during multiple group comparisons. Factors possibly associated with Vs of ≥1.55 m/s or with the development of HCC were assessed using stepwise logistic regression analysis. Statistical analysis was performed using the StatFlex version 5.0 for Windows (StatFlex, Osaka Japan). A two-sided p-value of <0.05 was considered significant.

Abbreviations

PNPLA3: Patatin-like phospholipase domain-containing 3; CHC: Chronic hepatitis C; LSM: Liver stiffness measurements; HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism; Vs: Velocity of a shear wave; BMI: Body mass index; IFN: Interferon; HCV: Hepatitis C virus; SVR: Sustained virological response; NAFLD: Nonalcoholic fatty liver disease; TE: Transient elastography; ARFI: Acoustic radiation force impulse; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: α-fetoprotein; PIVKA-II: Vitamin K absence or antagonist-II; NVR: Non-virological response.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KN and KY designed the project, carried out research and drafted the manuscript. SH, NK, YN, MM, TN, HS, TK, YT, MO, TK, TT, TN, NI, and KO contributed data collection. All authors read and approved the final manuscript.

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Review Article

Measurement of liver stiffness as a non-invasive method for diagnosis of non-alcoholic fatty liver disease

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Non-alcoholic fatty liver disease (NAFLD) is one of the major causes of liver disease worldwide. To detect early stages of NAFLD and start treatment or to monitor the changes in trials of new drugs, non-invasive diagnostic methods are needed, such as biochemical markers or liver stiffness measurement (LSM). LSM with transient elastography (TE) and acoustic radiation force impulse (ARFI) has been shown to be useful in NAFLD, although the cut-off values have varied among reports. Magnetic resonance elastography and real-time tissue elastography also can be useful for the diagnosis of NAFLD, although the number of studies is limited. Fibrosis is absent in 8–40% of patients with non-alcoholic steatohepatitis (NASH), making it difficult to diagnose NASH by LSM because LSM is usually associated with fibrotic stage. The presence of inflammation or hepatocyte ballooning may affect LSM and aid the diagnosis of NASH without fibrosis. However, obesity significantly increases the failure of LSM and its interference is more conspicuous in TE than in ARFI. The newly implemented XL probe of TE has overcome the difficulty to some degree. Nonetheless, the effects of obesity, hepatocyte ballooning, steatosis and inflammation on LSM values have not yet been adequately investigated, although they are likely to affect LSM values. Further studies are needed to establish the clinical utility of LSM in NAFLD.

Key words: acoustic radiation force impulse, magnetic resonance elastography, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, real-time tissue elastography, transient elastography

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is one of the major causes of liver disease worldwide. The worldwide prevalence of NAFLD has been estimated to range 6.3–33%, with a median of 20% in the general population. The prevalence of non-alcoholic steatohepatitis (NASH) has been estimated to range 3–5%.[1]

Practice guidelines by the American Association for the Study of Liver Diseases, American College of Gastroenterology and the American Gastroenterological Association categorized NAFLD histologically into non-alcoholic fatty liver (NAFL) and NASH. NAFL is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury, namely, hepatocytes ballooning.[2] NASH is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis.

The detection of steatosis usually depends on serum aminotransferase levels and imaging test such as ultrasonography, computerized tomography and magnetic resonance (MR). NAFL is generally benign, whereas NASH can progress to cirrhosis, liver failure and hepatocellular carcinoma. Thus, it is necessary to detect early stages of NASH and quickly initiate treatment. To detect the early stage of NASH, it is necessary to detect NASH with no or mild fibrosis. Because, till date, few medications are available to treat NAFLD,[2] large clinical trials of new medications are needed. For such clinical trials, detection of the early stage of NASH is necessary. Liver biopsy is the gold standard for diagnosis of NAFLD, although it is invasive and its accuracy for assessing steatosis and fibrosis is limited because of sampling errors and variations in interpretation among pathologists.[3] Non-invasive methods are needed to detect NASH and monitor NASH progression or resolution without the use of liver biopsy. There have been numerous reports on serum or genetic biomarkers and liver stiffness measurement (LSM) in the diagnosis of NAFLD.

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Liver stiffness measurement is a candidate non-invasive method for the diagnosis of NAFLD. Transient elastography (TE), acoustic radiation force impulse (ARFI), real-time TE (RTE) and/or MR elastography are available for LSM. These methods have been proven to be useful in the estimation of fibrotic stage in chronic viral hepatitis. The drawbacks reported include the effects of inflammation, obesity or lipid storage, which is a major factor contributing to NAFLD.[4–6] Obesity causes acquisition failure in TE[5] and ARFI.[6] High inflammatory activity causes high LSM values in chronic viral hepatitis.[4,7] High body mass index (BMI) causes lower ARFI values in chronic hepatitis C (CHC) with no or mild fibrosis.[8]

Non-alcoholic fatty liver disease is characterized by four pathological features: (i) lipid storage; (ii) fibrosis; (iii) inflammation; and (iv) hepatocyte injury. To discriminate NASH from NAFL, detection of fibrosis, inflammation or liver injury is required. Matteoni *et al.* classified NAFLD as follows: type 1, fatty liver alone; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning degeneration; and type 4, fat accumulation, ballooning degeneration and either Mallory–Denk body or fibrosis.[9] Type 3 and type 4 are considered as NASH. Kawaguchi *et al.* classified 543 patients with NAFLD by the Matteoni criteria into 102 patients (19%) with type 1, 75 (14%) with type 2, 31 (6%) with type 3 and 335 (62%) with type 4.[10] Thus, 8% (type 3) of patients with NASH had no fibrosis. The NASH Clinical Research Network (NASH CRN) proposed a NAFLD activity score (NAS), which was developed to measure changes in NAFLD during therapeutic trials and includes only features of active injury that are potentially reversible in the short term of clinical trials. NAS is defined as the sum of the scores for steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2); thus, ranging 0–8. Fibrosis is not included, because it is less reversible and is a result of disease activity.[11] The patients with a NAS of 2 or less have been considered as not having NASH and those with a NAS of 5 or more as having NASH, although the NASH CRN reported that the diagnosis is an unintended use of NAS and does not correspond to the diagnosis by the Pathology Committee of NASH CRN.[12] In their report, 12% of the patients with NASH with a NAS of 5 or more, 32% of borderline patients with NASH, or 7% of definite patients with NASH showed no fibrosis. Thus, 8–40% of patients with NASH were shown to have no fibrosis in the two studies. No increase in LSM may be detected in these patients because LSM is usually associated with fibrotic stage. It may be difficult to diagnose NASH in a patient with no fibrosis by LSM. The presence of inflammation or hepatocyte ballooning may affect LSM and aid the diagnosis of NASH without fibrosis.

Liver stiffness measurement may be affected either positively or negatively by factors other than fibrosis, such as steatosis, inflammation or liver injury, which are the major components of NASH. The effects of these factors on LSM are likely to influence the diagnosis of NASH by LSM, and therefore need to be assessed. LSM may be useful for detecting inflammation of NASH without fibrosis.

The currently available reports on LSM in the diagnosis of NAFLD will be presented and discussed in this review.

TE

LIVER STIFFNESS MEASUREMENT by TE was performed using FibroScan (EchoSens, Paris, France). FibroScan is equipped with a probe including an ultrasonic transducer and a vibrator (Fig. 1).[13] A vibration of mild amplitude and low frequency is transmitted from the vibrator placed on the body surface toward the liver through the intercostal space. The vibration induces an elastic shear wave that propagates through the liver tissue. The pulse-echo ultrasound acquisitions follow the propagation of the shear wave and determine its velocity. The velocity is directly related to tissue stiffness; the harder the tissue, the faster the shear wave propagates. LSM is calculated from velocity and expressed in kPa. LSM was performed after an overnight fast. Ten successful acquisitions were performed on each measurement and the median value was adopted as representative of LSM.

Several reports defined the cut-off values of fibrotic stages in NAFLD with the standard M probe of FibroScan (Table 1).[14–21] The cut-off values for F2, F3 and F4 were 6.6–7.8, 7.1–10.4 and 10.3–22.3 kPa, respectively. The cut-off values for each fibrotic stage varied among the reports.

Obesity, which is usually present in patients with NAFLD, causes failures of LSM acquisitions by TE. Several studies[14–18] reported failure rates ranging from 5%[14] to 9%.[16] Patients who had failed LSM acquisitions had higher BMI (35.6 ± 6.3 vs 28.0 ± 4.5 kg/m^2, $P < 0.001$) and waist circumferences (114 ± 14 vs 94 ± 12 cm, $P < 0.001$).[16] Valid LSM acquisitions were obtained in 98% of patients with BMI less than 30 kg/m^2 and 75% of patients with BMI of 30 kg/m^2 or higher. Thick subcutaneous adipose tissue probably

Table 1 Diagnostic performance of transient elastography for the detection of fibrosis in NAFLD

Study	Year	Disease	No. of patients	Probe	Fibrotic stage	Cut-off value (kPa)	AUROC	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Yoneda *et al.*[14]	2008	NAFLD	97	M	≥F2	6.6	0.86	88	74	79	85
					≥F3	9.8	0.90	85	81	64	93
					F4	17.5	0.99	100	97	75	100
Nobili *et al.*[15]	2008	NASH	52	M	≥F2	7.4	0.99	100	92	80	100
					≥F3	10.2	1.00	100	100	100	100
Wong *et al.*[16]	2010	NAFLD	246	M	≥F2	7.0	0.84	79	76	70	84
					≥F3	8.7	0.93	84	83	59	95
					F4	10.3	0.95	92	88	46	99
Lupsor *et al.*[17]	2010	NASH	72	M	≥F2	6.8	0.78	67	84	69	88
					≥F3	10.4	0.98	100	97	71	100
Petta *et al.*[18]	2011	NAFLD	169	M	≥F2	7.3	0.79	69	70	67	72
					≥F3	8.8	0.87	76	78	50	92
Mahadeva *et al.*[19]	2013	NAFLD	131	M	≥F3	7.1	0.77	70	67	38	89
					F4	11.3	0.95	88	89	34	99
Wong *et al.*[20]	2012	NAFLD	156	M	≥F2	7.0	0.83	79	64	62	80
					≥F3	8.7	0.87	83	78	58	93
					F4	10.3	0.89	81	83	35	98
		NAFLD	184	XL	≥F2	8.2	0.80	57	90	83	72
					≥F3	7.2	0.85	78	78	60	89
					F4	7.9	0.91	88	76	35	98
Myers *et al.*[21]	2012	NAFLD	75	M	≥F2	7.8	0.86	84	79	75	87
					F4	22.3	0.88	80	91	40	98
			75	XL	≥F2	6.4	0.85	81	66	61	84
					F4	16.0	0.95	100	91	40	100

AUROC, area under receiver–operator curve; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

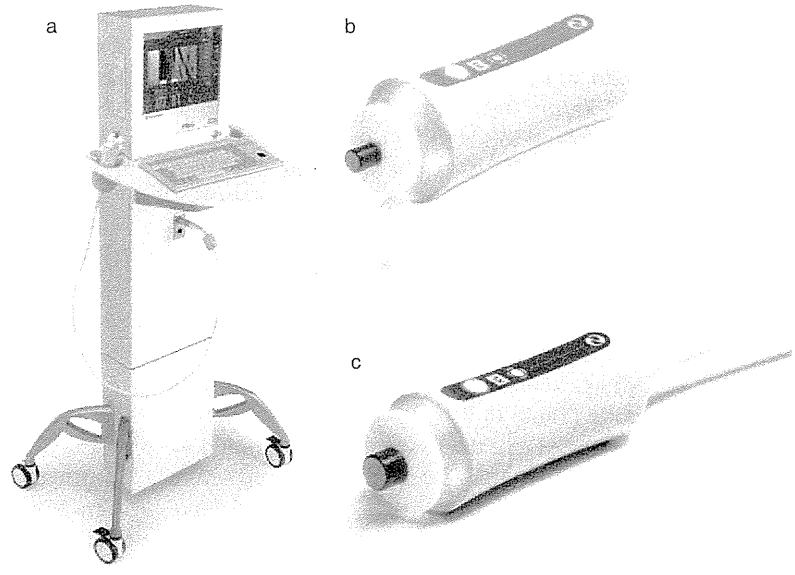

Figure 1 (a) FibroScan 502 for transient elastography. (b) M probe. (c) XL probe.

interferes with the transmission of the ultrasound and the elastic impulses, interfering with usable LSM acquisitions.

A new XL probe was recently proposed for use in obese patients (Fig. 1).[21,22] The XL probe differs from the standard M probe in that is emits a lower central ultrasound frequency (2.5 vs 3.5 MHz), in a higher vibration amplitude (3 vs 2 mm), from a larger tip diameter (12 vs 9 mm) and in an deeper explored region of interest (ROI) (3.5 vs 2.5 cm from the skin surface) to overcome the diagnostic difficulty caused by the interposition of thickened subcutaneous adipose tissue. The manufacturer recommends that the XL probe be used in patients with a skin-capsular distance of 2.5 cm or more.

Feasibility and performance of LSM are assessed based on failure (no valid measurements), successful LSM (≥10 valid measurements), success rate, interquartile range-to-median ratio (IQR/M) and reliable LSM (≥10 valid measurements, ≤30% IQR/M and ≥60% success rate). The XL probe achieves a higher frequency of successful LSM than the M probe (93% vs 65%,[21] 76% vs 45%[22] and 95% vs 81%,[20] respectively). Reliable LSM was obtained in 73% versus 50%[21] and 75% versus 67%[20] by the XL probe and the M probe, respectively. In the patients with BMI of less than 30 kg/m^2, successful LSM was obtained in 97% versus 92% by the XL probe and the M probe, whereas it was obtained in 93% versus 60% in those with BMI of 30 kg/m^2 or more.[2,20] In the patients with waist circumference of less than 102 cm, reliable LSM was obtained in 99% versus 91% by the XL probe and the M probe, whereas it was obtained in 59% versus 48% in those with waist circumference of 102 cm or more.[20] The rates of successful LSM by the XL probe and the M probe were 100% versus 92% in those with BMI of less than 30 kg/m^2, 95% versus 76% in BMI of 30 to less than 35 kg/m^2, 98% versus 50% in BMI of 35 to less than 40 kg/m^2, and 71% to 22% in body mass index of 40 kg/m^2 or more.[2,21] Thus, the XL probe has been proven to be feasible for patients with obesity. However, even the XL probe still has difficulty in patients with a skin-to-liver capsule distance of 3.4 cm or more [22] and extreme obesity (BMI ≥40 kg/m^2).[21]

The difference in cut-off values between the XL probe and the M probe is inappropriate. The cut-off values for the XL probe have been suggested to be lower than those for the M probe (Table 1).[21]

Gaia *et al.* reported that the LSM values determined by TE for NAFLD patients with advanced fibrosis and severe steatosis (>33%) were lower than expected and were similar to those of patients with mild fibrosis and mild steatosis. Gaia *et al.* concluded that severe steatosis may falsely reduce LSM by TE.[23] In contrast, Wong *et al.* reported that LSM is not affected by hepatic steatosis, necroinflammation or BMI.[16] Yoneda *et al.* reported that LSM values do not correlate with hepatic steatosis, but significant correlation is observed with inflammation, whereas the correlation was not confirmed by multivariate analysis.[14]

The reported cut-off values differ among various liver diseases, such as chronic hepatitis B and C, alcohol-related liver disease and NAFLD.[24] Likely explanations for the discrepancy include differences in the fibrotic staging system for different diseases, the quantity and character of fibrotic deposition (e.g. perisinusoidal/perivenular in NAFLD and periportal in viral hepatitis), the influence of non-fibrotic histological features (e.g. steatosis, ballooning and inflammation), and different distributions of fibrotic stages between diseases. Thus, optimal cut-off values should be determined specifically for NAFLD.

The M probe of FibroScan is equipped with a controlled attenuation parameter (CAP), which measures the ultrasound attenuation.[25] CAP has been shown to be useful for detection and semiquantification of liver steatosis in chronic liver disease from various etiologies. Thus, both fibrosis and steatosis can be simultaneously assessed by TE. However, the XL probe is not yet equipped with CAP.

ARFI

LIVER STIFFNESS MEASUREMENT by ARFI was performed using Siemens ACUSON S2000 (Siemens AG, Erlangen, Germany).[26] A region in the liver to be examined for elastic properties is targeted with a ROI cursor while performing B-mode imaging (Fig. 2).[27] Tissue at an ROI is mechanically excited using acoustic push pulses to generate localized tissue displacements. The displacements result in propagation of a shear wave away from the region of excitation, which is tracked using ultrasonic correlation-based methods. The maximal displacement is estimated for many ultrasound tracking beams laterally adjacent to the single push beam. By measuring the time to peak displacement at each lateral location, the shear wave propagation velocity can be reconstructed. The examination was performed on the right lobe of the liver. A measurement depth of 2–3 cm below the liver capsule was chosen. Ten successful acquisitions were performed on each patient and the results were usually expressed as shear wave velocity (SWV) in m/s, and the median value was

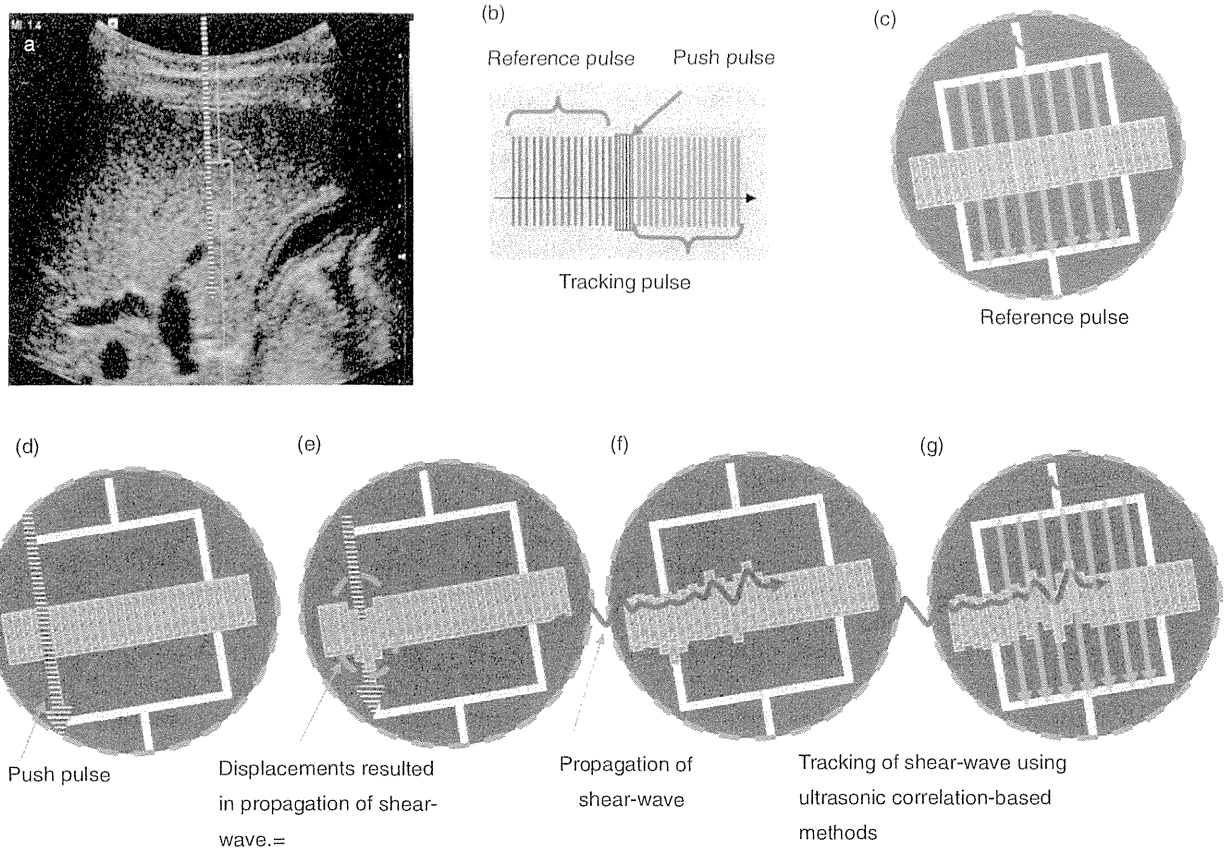

Figure 2 Acoustic radiation force impulse measurement. (a) A region in the liver to be examined for elasticity was targeted with a region of interest (ROI) cursor in B-mode imaging. The examination was performed on the right lobe of the liver. The measurement depth was 2–3 cm below the liver capsule. (b) Examination time course of acoustic radiation force impulse. (c) Reference pulses are used to establish a baseline position of the tissue prior to the acoustic radiation impulse. (d) Tissue at ROI was mechanically excited using acoustic push pulses to generate tissue displacements. (e) The displacements resulted in propagation of shear wave. (f) Propagation of shear wave. (g) The shear wave was tracked using ultrasonic correlation-based methods and its propagation velocity is obtained. Ten successful acquisitions were obtained and the median value was calculated. (Adapted from Saito[27] with permission.)

calculated. SWV is considered to be proportional to the square root of tissue elasticity.

Several reports defined the cut-off values of fibrotic stages in NAFLD with ARFI (Table 2).[28–31] The cut-off values for F2, F3 and F4 were 1.165, 1.48–2.06 and 1.635–1.9 m/s, respectively. The cut-off values discriminating NASH from NAFL were reported to be 1.105 m/s (F1)[31] or 1.3 m/s (steatosis with inflammation, without fibrosis).[30] The cut-off values for each fibrotic stage or for NASH varied considerably among reports.

Friedrich-Rust *et al.* compared the performance of ARFI on right and left lobes of liver with that of the M and XL probes of TE.[32] Successful LSM (≥10 valid measurements) were obtained in 100% of patients measured with ARFI in both lobes, 93% with the XL probe and 86% with the M probe. The rate of successful LSM of ARFI in both lobes is significantly higher than that of the M probe ($P = 0.0078$), but does not significantly differ from that of the XL probe. The success rate was 94 ± 10% with ARFI in the right lobe, 89 ± 14% with ARFI in the left lobe, 80 ± 27% with the M probe and 86 ± 22% with the XL probe. Thus, the ARFI success rate in the right lobe seems to be superior to the M probe.

Palmeri *et al.* performed three acquisitions at each of three locations to obtain a total of nine acquisitions.[29]

Table 2 Diagnostic performance of acoustic radiation force impulse for the detection of fibrosis in non-alcoholic fatty liver

Study	Year	Disease	No. of patients	Fibrotic stage	Cut-off value (m/s)	AUROC	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Yoneda *et al.*[28]	2010	NAFLD	54	≥F3	1.77	0.973	100	91	71	100
				F4	1.90	0.976	100	96	75	100
Palmeri *et al.*[29]	2011	NAFLD	172	≥F3	2.06†	0.90	90	90		
Guzman-Aroca *et al.*[30]	2012	NAFLD	32	NASH or fibrosis	1.3	0.899	85.0	83.3	89.4	76.9
Fierbinteanu Braticevici *et al.*[31]	2013	NAFLD	64	≥F1 (NASH)	1.105	0.867	76.70	71.40	84.62	60.00
				≥F2 (NASH)	1.165	0.944	84.80	90.30	90.32	84.85
				≥F3 (NASH)	1.480	0.982	86.40	95.20	90.48	93.02
				F4 (NASH)	1.635	0.984	91.70	92.30	73.33	97.96

†In the original published work of Palmeri *et al.*, the results of acoustic radiation force impulse were expressed as shear moduli ($\mu = cT^2\rho$ in kPa), which differs from Young's moduli ($E = 3\ \mu$ in kPa) usually quoted in published works involving transient elastography. cT represents shear wave velocity (m/s). ρ represents the constant density (1 g/cm^3).
AUROC, area under receiver–operator curve; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

After the elimination of spurious data, corrupted by excessive motion artifact, poor signal-to-noise ratio and inadequate imaging window, the data with an IQR/mean of more than 0.3 were considered too variable and were counted as failures. The rate of successful LSM was 100% in patients with BMI of less than 23 kg/m^2, 91% in those with BMI of 23 to less than 30 kg/m^2, 80% in those with BMI of 30–40 kg/m^2, and 58% in those with BMI of more than 40 kg/m^2.

Guzman-Aroca *et al.* evaluated 32 patients with morbid obesity (BMI > 49 kg/m^2 or BMI > 35 kg/m^2 with significant obesity-related comorbidities) by ARFI before bariatric surgery.[30] They reported that they had no problems in performing ARFI in patients with morbid obesity, although they obtained correct measurements in only 3–8 attempts.

Friedrich-Rust *et al.* compared area under the receiver–operator curve (AUROC) between ARFI and TE in patients in whom all ARFI, XL probe and M probe yielded successful acquisitions. No significant difference of AUROC was found between ARFI and TE for the diagnosis of significant fibrosis (0.84 for the XL probe vs 0.71 for ARFI of the right lobe, $P = 0.11$), for the diagnosis of severe fibrosis (0.83 for the XL probe vs 0.75 for ARFI of the right lobe, $P = 0.21$), for the diagnosis of cirrhosis (0.96 for TE combination score vs 0.94 for ARFI of the left lobe, $P = 0.67$) and for the diagnosis of steatohepatitis (0.71 for TE combination score vs 0.59 for ARFI of the left liver lobe, $P = 0.31$). Thus, the ability to diagnose using TE and ARFI seems to be equal, if a valid and reliable LSM is acquired.

Palmeri *et al.* reported that SWV by ARFI was not affected by the degree of hepatocyte ballooning or the amount of hepatic inflammation.[29] Fierbinteanu Braticevici *et al.* reported that ARFI measurements also correlated with the grade of inflammation ($r = 0.386$, $P < 0.001$) and steatosis ($r = -0.480$, $P < 0.001$); progressive decrease in SWV was proportional to steatosis severity.[31] Yoneda *et al.* also reported that SWV in the patients with NAFLD but no fibrosis was significantly lower than that in healthy volunteers ($P = 0.0058$).[28] Yoneda *et al.* noted that although ARFI velocity differed significantly between groups with different inflammatory activity, a stepwise change was not observed. Thus, it is probable that steatosis decreases SWV and inflammation increases SWV in NAFLD.

RTE

REAL-TIME ELASTOGRAPHY IS an imaging technique that can reveal the physical properties of tissue using conventional ultrasound probes; the Hitachi EUB-8500, EUB-900, HI VISION Ascendus, and HI VISION Preirus machines (Hitachi-Aloka Medical, Tokyo, Japan).[33] A region in the liver to be examined for elastic properties is targeted with an ROI cursor while performing B-mode imaging (Fig. 3). The ROI is divided into approximately 30 000 finite elements before compression. During the compression by the probe or heart beats, the displacement of each element is measured. In hard tissue, the amount of displacement is low, whereas in soft tissue the amount of displacement is high. The calculation of tissue elasticity distribution is performed in real time and the results are displayed as color-coded images with the conventional B-mode image in the background. In this manner, a large

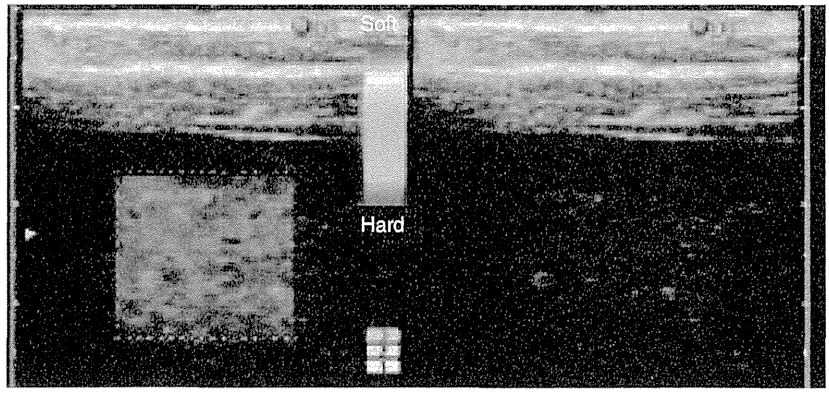

Figure 3 Real-time tissue elastography of a patient with F2 non-alcoholic fatty liver disease. A region in the liver to be examined for elastic properties is targeted with a region of interest (ROI) cursor while performing B-mode imaging. ROI is divided in approximately 30 000 finite elements before compression. During the compression by the probe or heart beats, the displacement of each element is measured. In hard tissue the amount of displacement is low, whereas in soft tissue the amount of displacement is high. The calculation of tissue elasticity distribution is performed in real time and the results are displayed as color-coded images with the conventional B-mode image in the background. (Courtesy of Dr Kazuhiko Hayashi, Department of Gastroenterology, Nagoya University.)

number of summarizing variables were obtained to characterize elastography. The final score was based on 10 summarizing variables selected to obtain high reproducibility. However, the methods used by the investigators to obtain the stiffness values differ among studies of NAFLD.

Tomeno *et al.* investigated the usefulness of the liver fibrosis index (LF Index) calculated using RTE in 27 patients with NAFLD and 93 patients with CHC. The LF Index showed significant correlation with fibrosis in patients with CHC ($P = 0.0102$) but not in patients with NAFLD ($P = 0.852$).[34]

Orlacchio *et al.* transformed all pixel data in the color-coded images into a histogram for quantification using a novel software, Elasto version 1.5.1 (developed by Hitachi Medical Systems, Tokyo, Japan), and tissue mean elasticity (TME) values were calculated (Table 3).[35] TME was described in arbitrary units (au) and was significantly correlated with fibrosis ($r = -0.75$, $P < 0.0001$) and steatosis ($r = -0.35$, $P = 0.02$) but not with the severity of inflammation. Multiple regression analysis showed that fibrosis was the only variable that significantly correlated with TME values ($P < 0.0001$). AUROC of TME for the fibrosis staging was 0.86 for F1 or more and was 0.92 for F2 or more.

Ochi *et al.* calculated the elastic ratio. ROI was simultaneously placed on small intrahepatic venous vessels with a diameter of less than 3 mm and on the hepatic

Table 3 Diagnostic performance of real-time tissue elastography for the detection of NASH in NAFLD

Study	Year	Disease	No. of patients	Diagnosis	Cut-off value	AUROC	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Orlacchio *et al.*[35]	2011	NASH	52	>F0	102 au	0.86	79	90	97	50
				≥F2	94 au	0.92	84	100	100	87
Ochi *et al.*[36]	2012	NAFLD	142	≥F1	2.47	0.838	64.9	96.9	98	54.4
				≥F2	2.67	0.853	86	88.6	87.8	82.5
				≥F3	3.02	0.878	88.2	91.5	83.3	94.2
				F4	3.36	0.965	100	85.6	55.2	100

au, arbitrary units; AUROC, area under receiver–operator curve; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

Table 4 Diagnostic performance of MR elastography for the detection of NASH in NAFLD

Study	Year	Disease	No. of patients	Diagnosis	Cut-off value (kPa)	AUROC	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Chen *et al.*[39]	2011	NAFLD	58	NASH	2.74	0.93	94	73	85	86
Kim *et al.*[40]	2013	NAFLD	142	≥F3	4.15	0.954	85	92.9		

AUROC, area under receiver–operator curve; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

parenchyma.[36] Subsequently, the ratio of the value in the intrahepatic venous small vessels was divided by the value in the hepatic parenchyma to generate the elastic ratio. The cut-off values for the elastic ratio for staging were determined in the training set and, using these cut-off values, the diagnostic accuracy of fibrotic stage in the validation set was 82.6–96.0% in all stages. Only portal fibrosis correlated with the hepatic elastic ratio by multivariate analysis.

There have been only a few reports on RTE of NAFLD and the methods used for obtaining stiffness values differ in each report. RTE would be expected to be useful for differential diagnosis of NASH, whereas further studies are needed to confirm the utility of RTE in NAFLD.

MR ELASTOGRAPHY

IN MR ELASTOGRAPHY, a drum-like acoustic passive driver is positioned over the liver and secured with a belt (GE Healthcare, Milwaukee, WI, USA). The passive driver is connected to an active acoustic driver system located outside of the scanner room via a tube. The active driver produces acoustic vibrations at 60 Hz, which are transmitted to the passive driver, which then transmits the vibrations into the body producing shear wave motion within the liver. A gradient-echo MR elastography sequence is used to acquire images showing shear wave propagation within the liver by encoding tissue motion into the phase of the measured MR signal.[37] The shear wave images are processed to produce images of hepatic stiffness (elastograms) using a direct inversion algorithm. The hepatic relative fat fraction (RFF) can also be measured using a two-point Dixon method with MR.[38]

Chen *et al.* reported that LSM was significantly higher in patients with NASH without fibrosis compared with patients with simple steatosis ($P = 0.028$) (Table 4).[39] LSM in patients with fibrosis was significantly higher than in patients with inflammation and no fibrosis ($P = 0.030$). LSM was significantly correlated with inflammation grade ($P = 0.0097$) and fibrotic stage ($P < 0.0001$) but not with RFF ($P = 0.52$). The cut-off value for discriminating NASH was 2.74 kPa.

Kim *et al.* reported that the cut-off value for advanced fibrosis (stage F3–F4) was 4.15 kPa.[40]

There have been only a few reports on MR elastography in NAFLD. Further studies are needed to confirm the utility of MR elastography in NAFLD, particularly the ability to discriminate NASH without fibrosis from NAFL. The interference of obesity in LSM acquisition also needs to be more thoroughly assessed.

CONCLUSION

REPORTS INVESTIGATING THE four procedures for LSM in the diagnosis of NAFLD have been reviewed, and they demonstrate both the benefits and weakness of the methodologies (Table 5).

Transient elastography and ARFI have been shown to be useful in NAFLD, although the cut-off values varied among the reports. It is still difficult to differentiate between NAFL and early stage NASH. There have only been a few reports on RTE and MR elastography for the diagnosis of NAFLD.

Approximately 8–40% of NASH patients have no fibrosis. It may be difficult to diagnose NASH in patients with no fibrosis by LSM because LSM is usually associated with fibrotic stage. The presence of inflammation or hepatocyte ballooning may affect LSM and aid the diagnosis of NASH without fibrosis.

Obesity significantly increases the failure of LSM and its interference is more conspicuous in TE than in ARFI. The newly invented XL probe of TE has overcome the difficulty to some degree.

The effects of obesity, hepatocyte ballooning, steatosis and inflammation on LSM values have not yet been adequately investigated.

Non-alcoholic fatty liver disease is a major health problem worldwide. LSM may become a clinical tool

Table 5 Benefits and weaknesses of the four procedures of liver stiffness measurement in NAFLD

Procedure	Benefits	Weaknesses
TE	Cut-off values for fibrotic stages were published in several reports, although they vary significantly. The grade of steatosis is assessed simultaneously by CAP.	Obesity causes failure of LSM. New XL probe has overcome the difficulty in some degree. Cut-off values for XL probe may be lower than those for M probe.
ARFI	Cut-off values for fibrotic stages were published in several reports, although they vary significantly. The apparatus is a conventional ultrasound machine and procedure is performed in B mode.	Failure of LSM by obesity is not conspicuous compared with TE.
RTE	The apparatus is a conventional ultrasound machine and procedure is performed in B mode.	A few reports have been published. The calculation of LSM has not been standardized.
MRE	Heavy machinery is needed. Hepatic relative fat fraction can also be measured.	Only a few reports have been published.

ARFI, acoustic radiation force impulse; CAP, controlled attenuation parameter; LSM, liver stiffness measurement; MRE, magnetic resonance elastography; NAFLD, non-alcoholic fatty liver disease; RTE, real-time tissue elastography; TE, transient elastography.

for mass screening and for monitoring the changes induced by treatment, replacing liver biopsy. Further studies are needed to establish the clinical utility of LSM in NAFLD.

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HEPATOLOGY

Effect of peginterferon alfa-2b and ribavirin on hepatocellular carcinoma prevention in older patients with chronic hepatitis C

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Abstract

Background and Aims: The population of patients chronically infected with hepatitis C virus (HCV) is aging, and the number of older patients with HCV-related hepatocellular carcinoma (HCC) is increasing. The purpose of this study was to elucidate the effects of peginterferon and ribavirin combination therapy on prevention of HCC in older patients with chronic hepatitis C (CH-C).

Methods: We compared the sustained virological response (SVR) and treatment discontinuation rates between older (≥ 65 years) and younger patients (< 65 years) among 1280 CH-C patients treated with peginterferon alfa-2b and ribavirin. Cumulative incidence of HCC was determined by Kaplan–Meier analysis, and factors associated with liver carcinogenesis were analyzed by Cox proportional hazards regression.

Results: Older patients had a significantly lower SVR rate and a significantly higher discontinuation rate of treatment than younger patients. Fifty patients developed HCC during median follow-up period of 47 months. Cox proportional hazards regression analysis indicated that the following were independent risk factors associated with the development of HCC: older age, male, advanced fibrosis, non-SVR in all patients: higher gamma-glutamyltranspeptidase, and non-SVR in older patients. Older patients who achieved SVR had a significantly reduced rate of HCC compared with those who did not achieve SVR, especially those who had gamma-glutamyltranspeptidase over 44 IU/L.

Conclusions: The SVR rate was lower and the combination therapy discontinuation rate was higher in older CH-C patients than in younger patients. However, older patients who achieved SVR had a markedly lower rate of HCC development compared with older patients who did not achieve SVR.

Introduction

Hepatitis C virus (HCV) infection is widespread, and often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The need for therapies to treat chronic HCV in older patients has intensified in Japan and is rising in the United States and other Western countries.[1] In addition, HCC has recently become a growing problem in patients with chronic hepatitis C (CH-C).

Interferon (IFN) treatment makes HCV remain in virological and biochemical remission with histological improvement in sustained virological responders who remain negative for serum HCV-RNA for 6 months.[2,3] Ribavirin is used in combination with IFN or peginterferon to treat CH-C, and combination therapy is reportedly more effective than IFN monotherapy, with a higher rate of HCV eradication.[4–7] Triple therapy with peginterferon, ribavirin, and telaprevir is now used for patients with CH-C, and this regimen has improved rates of HCV eradication; however, anemia, often severe in older patients with CH-C, and skin eruptions are significant side-effects.[8–11]

Several studies have shown that IFN monotherapy has comparable efficacy in older and younger patients with CH-C.[12,13] IFN

and ribavirin combination therapy has greater efficacy than IFN monotherapy.[4,6] However, since ribavirin reduces hemoglobin levels, higher number of patients need dose reductions. Patients over 65 years with genotype 1 and high HCV loads have a lower sustained virological response (SVR) rate than younger patients because of higher rates of ribavirin dose reduction and discontinuation due to ribavirin-related anemia.[14–16] In our previous study, we demonstrated that older patients have higher treatment discontinuation rates and lower SVR rates than younger patients. However, SVR was achieved in over half of elderly patients with genotype 2 and in elderly male patients with genotype 1, and low HCV-RNA concentrations.[15]

Eradication of HCV is important for patients with CH-C, but the ultimate treatment goal is prevention of liver cirrhosis and HCC. IFN therapy reduces the risk of HCC among virological or biochemical responders,[17–19] even in elderly patients with CH-C.[20–22] Veldt *et al.* reported that SVR with IFN-based therapy, including IFN, IFN plus ribavirin, and peginterferon plus ribavirin, reduced HCC development in patients with CH-C and liver cirrhosis.[23] Moreover, Morgan *et al.* reviewed that SVR among HCV-infected persons at any stage of fibrosis is associated with reduced HCC.[24] Several studies have shown that peginterferon and ribavirin prevent HCC in patients with CH-C, including cirrhosis.[25–29] However, there are no studies to date on the effect of peginterferon and ribavirin on HCC prevention focused on older patients with CH-C. In addition, no study has determined which older patient subpopulations with CH-C will benefit most from combination therapy in terms of HCC prevention.

The aim of this study was to elucidate the effects of combination therapy with peginterferon and ribavirin on prevention of HCC in older patients with CH-C.

Methods

Patients. This multicenter, retrospective cohort study included 1280 consecutive patients with CH-C who received peginterferon alfa-2b and ribavirin combination therapy at Nagoya University Hospital and its affiliated hospitals between December 2004 and December 2010. The ethics committee of Nagoya University Hospital approved the study protocol on the understanding that all data were coded to guarantee anonymity, and the study was performed in accordance with the 1975 Declaration of Helsinki.

Indications for treatment included age under 75 years, anti-HCV antibody positive status, and serum HCV-RNA levels greater than 100 000 IU/mL by a quantitative polymerase chain reaction (PCR) assay (Amplicor GT-HCV Monitor version 2.0; Roche Molecular Systems, Pleasanton, CA, USA) or 5 log IU/mL by a real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Diagnostics Japan, Tokyo, Japan) in the 12 weeks preceding treatment. In Japan, peginterferon and ribavirin combination therapy is only covered by medical insurance for patients with HCV-RNA levels greater than 100 000 IU/mL, considered a high viral load in Japan. Exclusion criteria included pretreatment hemoglobin levels < 10 g/dL, serum hepatitis B surface antigen positivity, autoimmune hepatitis, primary biliary cirrhosis, human immunodeficiency virus positivity, coexisting serious psychiatric or medical illness or alcohol abuse, and pregnancy. Alcohol intake was stopped at least 1 month before and during treatment. HCV genotypes were determined by PCR with genotype-specific primers previously described by Ohno *et al.*[30] Genotyping was performed at one centralized institution.

HCV genotype 1 and 2 patients were treated with 1.5 μg peginterferon alfa-2b (Pegintron, MSD, Tokyo, Japan) per kilogram of bodyweight subcutaneously once weekly for 48 and 24 weeks, respectively. When HCV eradication was detected between weeks 16 and 24 of treatment, treatment duration was prolonged up to 72 weeks for genotype 1 patients. Treatment was discontinued when a patient's hemoglobin concentration fell below 8.5 g/dL due to drug-induced hemolytic anemia, or when a patient's white blood cell (WBC) count fell below 1000/mm^3, neutrophil count fell below 500/mm^3, or platelet count fell below 50 000/mm^3. Oral ribavirin (Rebetol, MSD) was administered by standard protocol. Ribavirin was discontinued whenever peginterferon therapy was discontinued. Erythropoietin for anemia was not used because health insurance did not cover erythropoietin for this treatment in Japan.

Liver histology and definition of advanced fibrosis. Pretreatment liver biopsy specimens were performed at the start of treatment in 906 of 1280 patients, and analyzed for fibrosis on a scale of F0–F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis) and for necroinflammatory activity on a scale of A0–A3 (A0, no histological activity; A1, mild activity; A2, moderate activity; and A3, severe activity).[31] There is a selection bias to select patients who have received liver biopsy. Therefore, we used baseline parameter as marker for liver fibrosis. Platelet counts are often used as surrogate marker for liver fibrosis. Therefore, we determined the cut-off values of platelet counts for predicting F3–F4 by receiver–operator characteristics (ROC) analysis. Platelet counts < 141 000/μL were identified as cut-off values and the area under the curve was 0.795. Advanced fibrosis was defined as F3–F4 in patients who had liver biopsy and defined as platelet counts < 141 000/μL in patients who did not have liver biopsy.

Assessment of efficacy. Virological response was assessed by a qualitative HCV-RNA assay with a lower detection limit of 100 copies/mL (Amplicor HCV version 2.0; Roche Molecular Systems) or a quantitative HCV-RNA assay using a real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; lower limit of detection, 1.0 log IU/mL).[32,33] Based on the HCV-RNA values, SVR is defined as no HCV-RNA detected at the end of the 24-week follow-up period after treatment completion.

HCC surveillance and diagnosis. All patients underwent abdominal ultrasound or dynamic contrast-enhanced computed tomography (CT) to rule out pre-existing HCC at the start of treatment. HCC surveillance was conducted by ultrasonography every 4–6 months. Dynamic contrast-enhanced CT, dynamic contrast-enhanced magnetic resonance imaging, or CT-assisted angiography was performed when abdominal ultrasonography indicated a new lesion suspicious for HCC.

Comparison of characteristics and efficacy of treatment according to age. Patients were divided into two age groups: (i) older patients ≥ 65years old ($n = 254$) and (ii) younger patients < 65 years old ($n = 1026$). The following baseline parameters were compared between the two groups: gender; age; levels of aspartate aminotransferase, alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT), and hemoglobin; WBC count; platelet count; HCV genotype and viral load; histological activity; and fibrosis. In terms of HCV viral load, quantitative HCV-RNA results with Amplicor HCV version 2.0 was converted to real-time PCR-based results for HCV according to the reduction formula by Sizmann *et al.*[34] The SVR rates were calculated based on intention-to-treat and per-protocol analyses, and the peginterferon or ribavirin discontinuation rates were compared between the two age groups. In addition, we compared the cumulative incidence of HCC between patients who did and did not achieve SVR in the two age groups.

Factors associated with development of HCC. To identify factors that predict HCC development among patients treated with combination therapy, we first analyzed the factors independently associated with liver carcinogenesis by Cox proportional hazards regression in all patients, including gender (male *vs* female), age (older *vs* younger), baseline serum ALT, GGT, WBC count, hemoglobin, advanced fibrosis (advanced fibrosis *vs* non-advanced fibrosis), genotype, HCV-RNA level, and treatment efficacy (SVR *vs* non-SVR), and then analyzed in older patients.

Comparison of treatment efficacy among older patients who did and did not achieve SVR. To identify factors that predict SVR among patients treated with combination therapy, we first analyzed the baseline factors, outlined in the previous section, using a univariate model. Next, we identified the factors associated with SVR in combination therapy, including gender, baseline serum ALT, GGT, WBC count, hemoglobin, advanced fibrosis (advanced fibrosis *vs* non-advanced fibrosis), genotype, and HCV-RNA using a stepwise multivariate analysis with forward inclusion.

Comparison of treatment efficacy and cumulative incidence of HCC among older patients who did and did not achieve SVR. To identify older patients who may benefit especially from combination therapy, we determined factors associated with SVR using univariate analysis. We then determined factors associated with SVR in older patients treated with combination therapy by a stepwise multivariate analysis with forward inclusion. In addition, we compared the cumulative incidence of HCC among older patients who did and did not achieve SVR according to the platelet count and GGT cut-off values for predicting development of HCC based on ROC analyses.

Statistical analysis. Values are expressed as means ± SD. Between-group differences in mean quantitative values were analyzed using the Student's *t*-test, and differences in nonparametric data were analyzed using the Mann–Whitney *U*-test. Differences in proportions were tested with the chi-square test. Multiple logistic regression analysis was used to identify factors related to SVR. Cumulative incidence of HCC was determined by Kaplan–Meier analysis, and the factors independently associated with liver carcinogenesis were analyzed by Cox proportional hazards regression. Statistical analyses were performed using SPSS software version 20.0 (SPSS Japan Inc., Tokyo, Japan) for multiple logistic regression analysis, Kaplan–Meier analysis, Cox proportional hazards regression, and another analyses. All *P*-values were two-tailed, and $P < 0.05$ was considered statistically significant.

Results

Patient characteristics. The patients included 668 men and 612 women with an average age of 54.2 ± 12.1 years (mean ± SD). Patients aged ≥ 65 years comprised 19.8% of the patient population (254/1280). The baseline clinical characteristics of the two study groups are shown in Table 1. Compared with younger patients, older patients had significantly lower levels of ALT ($P = 0.0387$) and hemoglobin ($P < 0.0001$), as well as lower WBC and platelet counts ($P = 0.0010$ and $P < 0.0001$, respectively). HCV-RNA levels were also significantly lower in older patients ($P = 0.0359$). Fibrosis was more advanced in older patients ($P = 0.0001$).

Response to therapy and cumulative incidence of HCC. The intention-to-treat and per-protocol analyses both showed that the SVR rate in older patients was significantly lower than that in younger patients ($P < 0.0001$), and the treatment discontinuation rate was significantly higher in older patients ($P < 0.0001$) (Table 2). During median follow-up of 47 months, a total of HCC was found in 50 patients by surveillance ultrasonography, and diagnosed HCC as indicated in the Methods. No patients had symptoms and deterioration of liver function in each age cohort when HCC was found. Younger patients who achieved SVR had a significantly lower cumulative incidence of HCC than those who did not ($P = 0.003$) (Fig. 1a). However, due to a higher incidence of HCC in older patients, the difference in the cumulative incidence of HCC between older patients who achieved SVR and those who did not was larger than the difference between younger patients who did and did not achieve SVR ($P = 0.008$) (Fig. 1b).

Factors associated with hepatocarcinogenesis and SVR in all patients. Factors independently associated with development of HCC in all patients based on Cox proportional hazards regression analysis include age, advanced fibrosis, treatment efficacy, and gender (Table 3). Age and advanced fibrosis were independently associated with development of HCC.

Factors associated with hepatocarcinogenesis and SVR in older patients. Factors independently associated with development of HCC in older patients based on Cox proportional hazards regression analysis include GGT and treatment efficacy (Table 3).

To identify older patients who may benefit from achieving SVR, we examined the cumulative incidence of HCC according to GGT using cut-off values from the ROC curve for HCC. Among older patients with GGT < 44 IU/L, the cumulative incidence of HCC in

Table 1 Baseline clinical characteristics of patients treated with combination therapy

	All patients (n = 1280)	Older patients (n = 254)	Younger patients (n = 1026)	P-value
Gender (male/female)	668/612	123/131	545/481	0.180
Age (years)	54.2 ± 12.1	68.1 ± 2.8	50.7 ± 11.0	< 0.0001
AST (IU/L)	55.5 ± 44.0	59.0 ± 40.1	54.5 ± 44.9	0.2332
ALT (IU/L)	67.3 ± 64.3	59.9 ± 45.6	69.2 ± 68.0	0.0387
GGT (IU/L)	55.4 ± 71.2	47.7 ± 51.5	57.3 ± 75.1	0.0589
WBC (/μL)	5177.8 ± 1566.8	4889.2 ± 1313.7	5249.2 ± 1615.9	0.0010
Hemoglobin (g/dL)	14.0 ± 1.4	13.6 ± 1.3	14.1 ± 1.5	< 0.0001
Platelets (× 10^4/μL)	17.5 ± 6.3	15.4 ± 4.7	18.0 ± 6.6	< 0.0001
HCV-RNA (logIU/mL)	6.1 ± 0.7	6.0 ± 0.7	6.2 ± 0.7	0.0359
Genotype (1/2)	867/413	177/77	690/336	0.458
Activity (A0/A1/A2/A3)	48/491/338/32	10/97/77/10	38/394/261/22	0.3868
Fibrosis (F0–F1/F2/F3/F4)	567/213/109/17	94/56/36/5	473/157/73/12	0.0001

Patients were defined as two age groups: (i) older patients ≥ 65 years old and (ii) younger patients < 65 years old.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltranspeptidase; HCV-RNA, hepatitis C virus RNA; WBC, white blood cell.

Table 2 Efficacy of combination therapy

	All patients (n = 1280)	Older patients (n = 254)	Younger patients (n = 1026)	P-value
SVR rate (ITT)	54.8 (701/1280)	38.2 (97/254)	58.9 (604/1026)	< 0.0001
SVR rate (PP)	62.9 (687/1092)	46.7 (92/197)	66.5 (595/895)	< 0.0001
Discontinuation rate of treatment	14.7 (188/1280)	22.4 (57/254)	12.8 (131/1026)	< 0.0001

ITT, intention-to-treat; PP, per-protocol; SVR, sustained virological response.

those who did not achieve SVR was higher than of those who achieved SVR, but this difference was not significant (Fig. 2a). However, in older patients with GGT ≥ 44 IU/L who achieved SVR, there was a marked reduction in the development of HCC compared with the older patients with GGT ≥ 44 IU/L who did not achieve SVR (older patients with GGT < 44 IU/L, $P = 0.265$; older patients with GGT ≥ 44 IU/L, $P = 0.020$, log–rank test) (Fig. 2b).

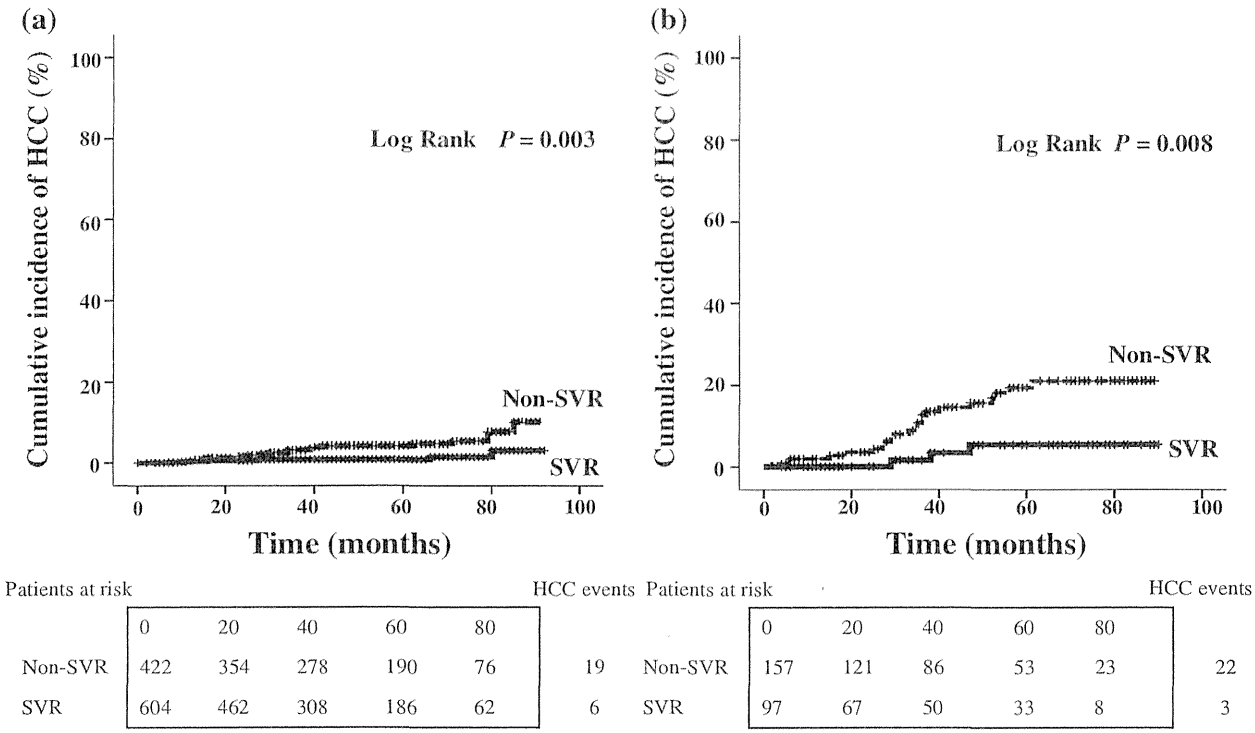
Next, we analyzed which older patients were more likely to achieve SVR. At first, we identified factors associated with SVR by univariate analysis. Among older patients, the ratio of males who achieved SVR was higher than among those who did not ($P = 0.0017$). The baseline HCV viral load in patients who achieved SVR was significantly lower than that in patients who did not achieve SVR ($P < 0.0001$). There was a higher proportion of genotype 2 patients who achieved an SVR than genotype 2 patients who did not ($P = 0.0003$) (Table 4). Factors associated with SVR in combination therapy were determined by multivariate analysis. HCV-RNA, gender, and genotype were significantly associated with SVR in older patients (Table 4).

Discussion

IFN-based therapy, including combination therapy with ribavirin, has improved the SVR rate in patients with CH-C and liver cirrhosis. However, there have been several reports of HCC occurring in patients despite achieving SVR, so the need for long-term follow-up remains among patients who achieve SVR.[35–37] HCC remains life-threatening in patients with eradicated HCV. Achieving SVR is important to reducing hepatic inflammation and histological improvement. However, the ultimate goal of treatment for patients with CH-C is to prevent the development of liver cirrhosis and HCC. It is important to evaluate indications for treatment in terms of HCC prevention in CH-C patients. This study provides data on HCC incidence after combination therapy consisting of peginterferon and ribavirin in CH-C patients, including older patients, who have a high incidence of HCC.

Due to the high incidence of HCC in older patients, the cumulative incidence of HCC in older patients showed significant reduction among older patients who achieved SVR compared with those who did not achieve SVR, and this decrease was more distinct than in younger patients in this study. Previous reports have shown that IFN monotherapy reduces the risk of HCC development,[38,39] even in patients ≥ 60 years if they achieve SVR.[20–22] These are all treated with IFN monotherapy. Given the current aging trend in CH-C patients, older patients are often defined as patients ≥ 65 years of age. However, there are no reports on the effects of peginterferon and ribavirin on the development of HCC in patients over 65 years. This is the first report that peginterferon alfa-2b plus ribavirin was associated with a significant reduction in the development of HCC in patients ≥ 65 years if they achieved SVR.

In this cohort, factors associated with HCC development among older patients included GGT and non-SVR status. GGT is a heterodimeric glycoprotein that catalyzes the transpeptidation and hydrolysis of the gamma-glutamyl group of glutathione and



Patients at risk	0	20	40	60	80	HCC events
Non-SVR	422	354	278	190	76	19
SVR	604	462	308	186	62	6

Patients at risk	0	20	40	60	80	HCC events
Non-SVR	157	121	86	53	23	22
SVR	97	67	50	33	8	3

Figure 1 Cumulative incidence of HCC after peginterferon alfa-2b and ribavirin in patients who achieved SVR (solid line) or did not achieve SVR (dashed line) in younger patients < 65 years old (a) and older patients ≥ 65years old (b). The number of patients at risk and HCC events at each time point are shown below the graphs. HCC, hepatocellular carcinoma; SVR, sustained virological response.

Table 3 Factors associated with development of HCC

All patients				
Variable	Category	Hazard ratio	95% CI	*P*-value
Age	Younger	1	1.927–6.369	< 0.0001
	Older	3.504		
Advanced fibrosis	Non-advanced fibrosis	1	1.601–5.308	< 0.0001
	Advanced fibrosis	2.915		
Treatment efficacy	SVR	1	2.209–9.942	< 0.0001
	Non-SVR	4.686		
Gender	Female	1	1.283–4.495	0.006
	Male	2.402		
Older patients				
Variable	Category	Hazard ratio	95% CI	*P*-value
GGT	< 44	1	2.869–20.269	< 0.0001
	≥ 44	7.626		
Treatment efficacy	SVR	1	1.175–14.882	0.027
	Non-SVR	4.181		

CI, confidence interval; GGT, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; SVR, sustained virological response.