Table 2. Risk factors for HCC-related death evaluated by univariate/multivariate Cox proportional hazard regression

	Univariate		Multivariate	
Parameter	**HR (95% CI)**	***p* value**	**HR (95% CI)**	***p* value**
Age (year)	1.02 (0.95–1.10)	0.60		
Female	1.45 (0.56–3.77)	0.44		
Hepatitis B	1.37 (0.18–10.3)	0.76		
MtCK >19.4 (U/L)	5.03 (1.93–13.1)	<0.001	2.32 (1.03–5.25)	0.042
Albumin	0.15 (0.05–0.44)	<0.001	0.26 (0.09–0.71)	0.009
AST	1.02 (1.01–1.03)	<0.001	1.01 (1.00–1.02)	0.028
ALT	1.01 (0.99–1.02)	0.13		
GGT	1.00 (0.98–1.01)	0.45		
Total bilirubin	3.23 (1.98–5.29)	<0.001	1.72 (0.97–3.04)	0.064
AFP >100 (ng/dL)	2.28 (0.84–6.18)	0.11		
DCP >80 (mAU/mL)	2.74 (0.99–7.45)	0.59		
Platelet	0.83 (0.71–0.97)	0.017	0.89 (0.76–1.04)	0.14
Prothrombin time	1.32 (1.11–1.57)	0.002	0.91 (0.70–1.17)	0.45
Liver stiffness	1.02 (0.98–1.04)	0.25		

Reduction of uMtCK expression in HCC cells led to the inhibition in their proliferation, migration and invasion. The similar effects of inhibition of uMtCK expression were reported in Hela cells[29] and breast cancer cells.[17] This finding may be in agreement with the notion that the creatine kinase system is generally essential for the control of cellular energetics in tissues or cells with high and fluctuating energy requirements.[37] Indeed, overexpression has been reported for different creatine kinase isoforms in different types of cancer and has provided a more general growth advantage to solid tumors.[37,38] Overexpression of uMtCK in different Hodgkin-derived cell lines has been described as a marker for poor prognosis.[39] Increased uMtCK levels in cancer cells might be a part of metabolic adaptation of those cells to perform high growth rate under oxygen and glucose restriction as typical for many cancers; it could help to sustain energy turnover, but would be also protective against stress situations such as hypoxia and possibly protect cells from death.[40] Nonetheless, these *in vitro* findings raise the possibility that high expression of uMtCK in HCC may be associated with its active growth and metastasis.

Then, we performed a follow-up study of the HCC patients, with whom we showed the increased serum MtCK activity.[16] Among the entire HCC patients in the previous study, we enrolled the patients who underwent RFA with curative intent to examine the potential association between serum MtCK activity and prognosis in this study. In the previous report, serum MtCK activity was also enhanced in the patients with liver cirrhosis compared to healthy control, although less prominent than in those with HCC and liver cirrhosis,[16] suggesting that background liver status of HCC may also affect serum MtCK activity. In this context, because RFA with curative intent was performed on patients without advanced liver damages such as high serum total bilirubin concentration, low platelet counts or massive ascites,[33] the potential association between serum MtCK activity and prognosis of HCC patients could be assessed with less bias from background liver status. Furthermore, of note, HCC patients treated with RFA had no extended tumor lesions, that is, three or fewer lesions, each 3.0 cm in diameter.[33] As a result, the HCC patients with higher serum MtCK activity had a significantly poorer prognosis than those with lower serum MtCK activity on a survival analysis, and higher serum MtCK activity was retained as a significant risk for HCC-related death on multivariate analysis. Thus, in line with the current *in vitro* findings, it is suggested that HCC with increased uMtCK expression may have highly malignant potential.

In conclusion, high uMtCK expression in HCC may be caused by hepatocarcinogenesis *per se* but not by loss of mitochondrial integrity, and associated with highly malignant potential, where ASB9 could be one of the regulators of uMtCK expression. In the clinical setting, higher serum MtCK activity was associated with a poorer prognosis of HCC, suggesting that HCC with high serum MtCK activity should be thoroughly treated when considered to be curative.

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Alcohol and Smoking Affect Risk of Uncomplicated Colonic Diverticulosis in Japan

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Abstract

Colonic diverticula are located predominantly on the right side in Asia and on the left side in Europe and the United States. Factors associated with uncomplicated colonic diverticulosis and its distribution pattern have been unknown. Our aims are to investigate the prevalence and risk factors for uncomplicated colonic diverticulosis. We conducted a prospective cross-sectional study in adults who underwent colonoscopy. Alcohol, alcohol related flushing, smoking, medications, and comorbidities were assessed by interview on the colonoscopy day. Alcohol consumption was categorized as nondrinker, light (1–180 g/week), moderate (181–360 g/week), and heavy (≥361 g/week). Smoking index was defined as the number of cigarettes per day multiplied by the number of smoking years and categorized as nonsmoker, <400, 400–799, and ≥800. A total of 2,164 consecutive patients were enrolled. Overall, 542 patients (25.1%) had uncomplicated colonic diverticulosis located on the right side (50%), bilaterally (29%), and on the left side (21%). Univariate analysis revealed age, male, smoking index, alcohol consumption, aspirin use, anticoagulants use, corticosteroid use, hypertension, and atherosclerotic disease as factors significantly associated with diverticulosis. Alcohol related flushing was not associated with the disease. Multivariate analysis showed increasing age ($P<0.01$), increasing alcohol consumption ($P<0.01$) and smoking ($P<0.01$), and atherosclerotic disease ($P<0.01$) as significantly associated factors. Alcohol and smoking were associated with right-sided and bilateral diverticula. In conclusion, one in four Japanese adults have colonic diverticulosis (50% right-sided). Age, alcohol consumption, and smoking were found to be significant risk factors for uncomplicated colonic diverticulosis, particularly right-sided and bilateral.

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Introduction

Colonic diverticulosis shows geographic variation in both prevalence and pattern. Diverticulosis is rare in Africa and Asia, but common in the United States, Europe, and Australia [1–4]. The anatomic distribution pattern of diverticulosis is predominantly left-sided in the West and right-sided in Asia [1,4,5].

In Japan, the prevalence of colonic diverticulosis was 2.1% in the 1960s, but increased to 28% by 1997 [6–9]. Recent studies have shown that the current prevalence in Korea is 12% [10]. The increased prevalence of diverticulosis in Asian countries suggests that environmental and lifestyle factors play an important role in its pathogenesis [1,4,10].

Because asymptomatic colonic diverticulosis has potential to cause serious complications, such as diverticular bleeding or diverticulitis, it is crucial to understand the true prevalence and risk factors of the disease to prevent associated morbidity and mortality. However, the exact risk factors for uncomplicated colonic diverticulosis other than age remain unknown. Although constipation and low-fiber diet have been widely accepted as etiological factors for uncomplicated diverticulosis [1,4,11,12], a recent study showed that a high bowel movement frequency and a high-fiber diet were associated with a higher prevalence of diverticulosis [13]. Furthermore, a prospective cross-sectional study in Korea and the United States revealed alcohol consumption as a new risk factor for uncomplicated diverticulosis [10,14]. Therefore, further exploration of diverticulosis risk factors is needed.

The widespread use of colonoscopic examination has enabled increased detection of colonic diverticulosis [10], but few studies have reported on the specific factors associated with diverticulosis, especially in Asia [6,10]. To investigate the prevalence and to identify possible associated factors or risk factors of diverticulosis, we analyzed comprehensive data obtained from a prospective colonoscopy-based study that collated detailed information on smoking, alcohol consumption, comorbidities, and medications.

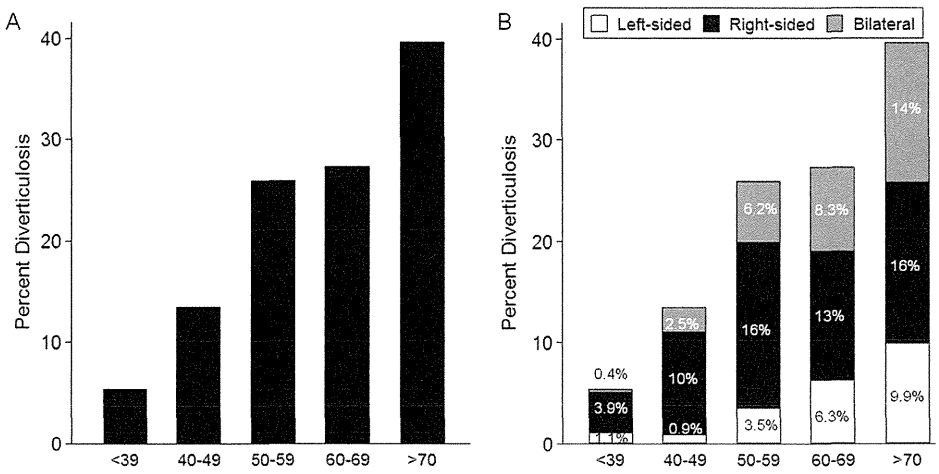

Figure 1. Prevalence of diverticulosis (A) and anatomic distribution (B) by age category (n = 2,164). Colonic diverticulosis increased with age (A). The prevalence of right-sided diverticula was high at for younger age, while left-sided and bilateral types increased with age (B).
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Materials and Methods

Study design, setting, and participants

We conducted a prospective cross-sectional single-center study in adults who underwent diagnostic colonoscopy between September 2009 and July 2012 at the endoscopy unit of the National Center for Global Health and Medicine (NCGM). The NCGM is an emergency hospital with 900 beds located in metropolitan Tokyo, Japan. The institutional review board at NCGM approved this study (No. 750) and all clinical procedures conformed to Japanese and International ethical guidelines (Declaration of Helsinki). All patients gave informed written consent prior to enrolment. No ethical problems exist with regard to the publication of this manuscript. We used anonymized data from patient medical records.

Inclusion criteria were as follows: (1) >18 years old; (2) Japanese nationality; (3) independence in activities of daily living; (4) able to understand written documents; (5) able to write; (6) asymptomatic patients who needed examination for colorectal cancer due to increasing tumor marker and/or fecal occult blood test results and/or abnormal findings on abdominal ultrasonography, computed tomography (CT), positron emission tomography-computed tomography (PET-CT), magnetic resonance imaging (MRI); or patients who wanted screening for colorectal cancer. Exclusion criteria were as follows: (1) patients who did not provide informed consent; (2) patients in whom total colonoscopy could not be performed; (3) and history of colon resection; (4) acute colonic diverticular bleeding or diverticulitis; (5) severe, continuous, or intermittent gastrointestinal (GI) symptoms such as frequent watery diarrhea and hematochezia within one week of onset to determine appropriate medical treatment. All inclusion and exclusion criteria were fulfilled before patients were enrolled.

Variables, Data sources, and Measurement

After informed consent was obtained, a detailed questionnaire was completed at the endoscopy unit on the same day as pre-colonoscopy. Patients were asked about their 1) lifestyle habits, 2) medications, and 3) comorbidities in a face-to-face interview with medical staff. For medication history, prescriptions and medical records were reviewed in addition to information provided by the patients to avoid omissions.

Patients were asked the following four questions regarding alcohol consumption: "*Do you drink alcohol?*", "*What types of alcohol do you usually drink; for example, beer, shochu, sake, wine, gin, vodka, whiskey, tequila, or brandy?*", "*How many days per week do you drink alcohol?*", and "*How many glasses of about 180 ml of alcohol do you usually drink per day?*" Then, alcohol consumption was calculated and subjects were categorized as nondrinker, light drinker (1–180 g/week), moderate drinker (181–360 g/week), and heavy drinker (≥361 g/week). Duplicate data were allowed.

The flushing questions consisted of the following two items: "*Do you have a tendency to develop facial flushing immediately after drinking a glass of about 180 ml of beer?*" and "*Did you have a tendency to develop facial flushing immediately after drinking a glass of beer in the first one or two years after you started drinking alcohol?*" For both questions, the choice of answers was "*yes*", "*no*", and "*unknown*". If a subject answered yes to either question, they were considered to be deficient in acetaldehyde dehydrogenase 2 (ALDH2) [15].

The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years. Then, smoking index was categorized as nonsmoker, <400, 400–799, and ≥800.

Patients were asked about regular use of aspirin, anticoagulants, and oral corticosteroids (prednisolone, methylprednisolone, betamethasone, dexamethasone, or hydrocortisone). The survey form included photographs of these oral drugs, which are approved in Japan. Regular use of medication was defined as oral administration starting at least 1 year before the interview.

Evaluated comorbidities were hypertension, atherosclerotic vascular disease including diabetes mellitus and dyslipidemia, coronary heart disease, and chronic renal failure. Diabetes mellitus, dyslipidemia, and hypertension were considered present in patients taking specific drugs. Chronic renal failure was considered present in patients on hemodialysis or peritoneal dialysis, or with serum creatinine levels ≥2.0 mg/dl.

An electronic high-resolution video endoscope (model CFH260; Olympus Optical, Tokyo, Japan) was used for diagnosis of colonic diverticula. Intestinal lavage for endoscopic examination was performed using 2 L of solution containing polyethylene glycol. If diverticula were observed within the colon, their location type was recorded in the electronic endoscopic database. Distribution type was defined as follows: right-sided, involving the splenic flexure,

Table 1. Characteristics in patients with or without colonic diverticulosis on univariate analysis (n = 2,164).

Variables	All cases (n = 2,164)	With Diverticulosis (n = 542)	Without diverticulosis (n = 1,622)	P
Mean Age (SD), years	58 (14)	56 (15)	65 (11)	<0.01
<39	280 (13)	15 (2.8)	265 (16)	
40–49	320 (15)	43 (7.9)	277 (17)	
50–59	374 (17)	97 (18)	277 (17)	
60–69	685 (32)	187 (35)	498 (31)	
>70	505 (23)	200 (37)	305 (19)	<0.01
Sex (Male)	1,356 (63)	364 (67)	992 (61)	0.01
Smoking index*				
Nonsmoker	1,056 (49)	214 (39)	842 (52)	
<400	533 (25)	93 (17)	440 (27)	
400–799	319 (15)	114 (21)	205 (13)	
>800	256 (12)	121 (22)	135 (8.3)	<0.01
Alcohol				
Non-drinker	856 (40)	142 (26)	714 (44)	
Drinker	1,308 (60)	400 (74)	908 (56)	<0.01
Alcohol consumption				
Non-drinker	856 (40)	142 (26)	714 (44)	
Light drinker (1–180 g/week)	983 (45)	270 (50)	713 (44)	
Moderate drinker (181–360 g/week)	207 (9.6)	69 (13)	138 (8.5)	
Heavy drinker (≥361 g/week)	118 (5.5)	61 (11)	57 (3.5)	<0.01
Alcohol Flusher				
Non-flusher or unknown	1708 (79)	434 (80)	1274 (79)	
Flusher	456 (21)	108 (20)	348 (21)	0.45
Medication				
Regular aspirin use	206 (9.5)	80 (15)	126 (7.8)	<0.01
Regular anticoagulants use	103 (4.8)	40 (7.4)	63 (3.9)	<0.01
Regular corticosteroid use	170 (7.9)	26 (4.8)	144 (8.9)	<0.01
Comorbidity				
Hypertension	745 (34)	264 (49)	481 (30)	<0.01
Atherosclerotic vascular disease	644 (30)	223 (41)	421 (26)	<0.01

Categorical variables are reported as n (%).
*The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years.
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transverse or proximal colon; left-sided, involving the descending or distal colon; or bilateral, involving the entire colon.

Statistical analysis

Patients with colonic diverticula were defined as subjects, and those without colonic diverticula were defined as controls, and the relationships between colonic diverticula and clinical factors were examined. To determine risk factors for colonic diverticula, we estimated the odds ratio (OR) and 95% confidence interval (CI). Pearson's Chi-squared test was used to determine the univariate association between each variable and the presence of diverticulosis. In multivariate analysis, we used a multiple logistic regression model. The Cochran–Armitage test was used to identify the trend between each variable and the presence of diverticulosis. A value of $P<0.05$ was considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, College Station, Texas, USA).

Results

Participants and prevalence

During the study period, 2,319 patients participated in medical interviews. Of them, 91 could not undergo total colonoscopy and 64 had a history of colorectal resection. Ultimately, 2,164 consecutive patients comprising 542 patients with uncomplicated colonic diverticula were enrolled. The overall prevalence of colonic diverticulosis was 25.0%. Diverticula were located predominantly in the right-side of the colon in 50.0% (n = 271), bilaterally in 29.3% (n = 159), and in the left-side in 20.7% (n = 112) of cases. The prevalence of colonic diverticulosis increased with age (Figure 1A). The prevalence of right-sided diverticula was significantly higher in younger patients than in older patients (<39 vs 40–59 years, $p<0.01$; 40–59 vs 50–59 years, $p = 0.02$), and tended to increase significantly ($P<0.01$ for trend) with age. Similarly, that for left-sided and bilateral types also tended to increase significantly ($P<0.01$ for trend) with age (Figure 1B). There were 223 patients (10.3%) who underwent on

Table 2. Factors associated with colonic diverticulosis on multivariate analysis.

Variables	Odds ratio (95% CI)	P
Age	1.1 (1.0–1.1)	<0.01
Sex		
Female	1 (referent)	
Male	1.0 (0.74–1.2)	0.75
Smoking index*		
Nonsmoker	1 (referent)	
<400	0.90 (0.66–1.2)	0.47
400–799	1.7 (1.3–2.4)	<0.01
>800	1.8 (1.3–2.5)	<0.01
Alcohol consumption		
Non-drinker	1 (referent)	
Light drinker (1–180 g/week)	2.2 (1.7–2.8)	<0.01
Moderate drinker (181–360 g/week)	2.7 (1.8–4.0)	<0.01
Heavy drinker (≥361 g/week)	5.6 (3.6–8.8)	<0.01
Alcohol Flusher		
Non-flusher or unknown	1 (referent)	
Flusher	0.87 (0.66–1.1)	0.30
Medication		
Regular aspirin use		
No	1 (referent)	
Yes	1.1 (0.75–1.5)	0.73
Regular anticoagulants use		
No	1 (referent)	
Yes	1.4 (0.88–2.2)	0.16
Regular corticosteroid use		
No	1 (referent)	
Yes	0.69 (0.43–1.1)	0.11
Comorbidity		
Hypertension		
No	1 (referent)	
Yes	1.2 (0.95–1.5)	0.13
Atherosclerotic vascular disease		
No	1 (referent)	
Yes	1.4 (1.1–1.8)	<0.01

*The smoking index was evaluated among ever and daily smokers and was defined as the number of cigarettes per day multiplied by the number of smoking years.

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CT, PET-CT, or MRI. No significant differences in the prevalence of colonic diverticulosis were noted between the group with abnormal imaging findings and the group with normal imaging findings in 23.3% (n = 17/73) and 22.0% (n = 33/150) (P = 0.83) of cases, respectively.

Risk factors

Table 1 shows patient characteristics. On univariate analysis, age, male, smoking index, alcohol consumption, regular aspirin use, regular anticoagulants use, regular corticosteroid use, hypertension, and atherosclerotic disease were significantly associated with diverticulosis. On multivariate analysis, increasing age, increasing alcohol consumption and smoking, and atherosclerotic disease were significantly associated with diverticulosis (Table 2). Relative risk tended to increase with age, smoking index, and the amount of alcohol consumed (Table 2).

Distribution type and factors

Right-sided and bilateral diverticula increased significantly in line with alcohol consumption (P<0.01 for trend) (Figure 2A), while left-sided diverticula were not significantly (P = 0.60) associated with alcohol consumption (Figure 2A).

Distribution type of colonic diverticula increased significantly in line with smoking index (left-sided: P<0.01; right-sided: P<0.01; and bilateral diverticula: P<0.01; respectively for trend) (Figure 2B).

Discussion

In this colonoscopy-based study, we demonstrated that older age and alcohol consumption are strong risk factors for uncomplicated colonic diverticulosis, and the risk increases in line with the amount of alcohol consumed. Furthermore, patients with high pack-years of smoking, hypertension, and atherosclerotic vascular disease were found to be predisposed to colonic diverticulosis. The prevalence of right-sided and bilateral diverticula increased significantly as alcohol consumption and smoking increased.

Several studies have shown that the prevalence of colonic diverticulosis in Japan increased to 28% by 1997 [6–9]. Although no available data were available for the 2000s, we found that the prevalence for this period was 25%. To our knowledge, this is the first prospective study to identify the prevalence of colonic diverticulosis in Japan based on colonoscopic findings because most previous studies used barium enema [6–9]. Colonoscopy is used worldwide as a standard tool for the detecting colonic cancer and diverticulosis [10], but it can miss diverticula, especially those in the left-sided colon [16]. Thus, the prevalence of diverticula determined in this study is likely to be lower than the actual prevalence.

Age has been found to be an important risk factor for colonic diverticulosis. It has been suggested that patients with diverticular disease have greater rates of collagen cross-linking [17]. In addition, abnormal thickness of muscles of colonic wall, including collagen cross-linking, is promoted by abnormal colonic movement due to a lack of dietary fiber and results in increased intraluminal colonic pressure or fragility of the thickened muscles due to intraluminal pressure changes with age [3,4].

Consistent with past studies [4,5,8,9], the diverticula in our subjects first developed on the right-sided colon and extended to the left-sided and bilateral colon with aging. Right-sided diverticula in the Japanese has been considered to be of congenital origin [4], thus identification of factors associated with the right-sided type is important to understand the development of colonic diverticula. Why diverticulis is predominantly right-sided in Asian people and rarely so in other populations, who have the same risk factors as those identified in this study, is unclear. It is possible, however, that differences in the sensitivity of the colon to environmental factors are due to variations in characteristics such as the length and muscle thickness of the colon, body weight, and the structure of the neural and humoral systems [4].

In the present study, alcohol intake and amount were not only associated with the entire colonic diverticula but specifically with right-sided diverticula. Song et al. [10] revealed that alcohol drinkers were two times (OR: 2.2) more likely to develop diverticulosis than nondrinkers when assessed by multivariate analysis in a colonoscopy-based study. Sharaha et al. [14] recently

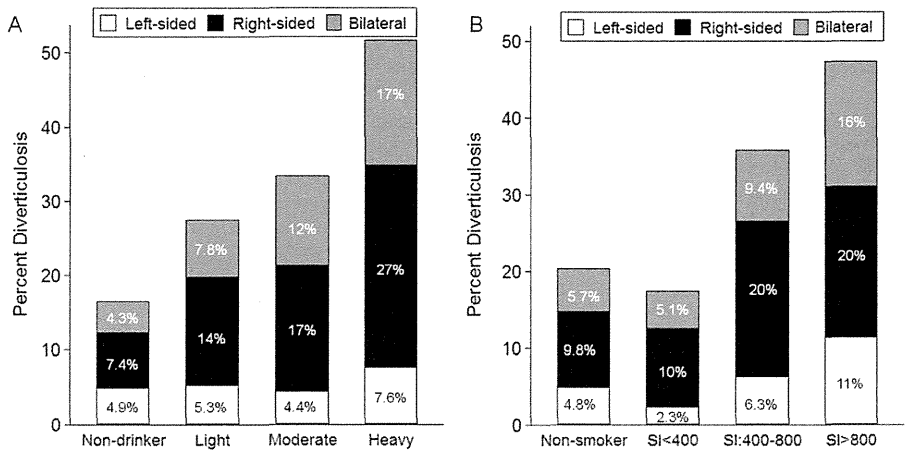

Figure 2. Prevalence of diverticulosis and anatomic distribution by alcohol consumption (A) and smoking index (B) (n = 2,164). Right-sided and bilateral diverticula increased significantly in line with amount of alcohol consumption (A). All distribution types of colonic diverticula increased significantly in line with smoking index (SI) (B).
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conducted a prospective colonoscopy-based study and found that the OR for diverticula was 1.96 with occasional alcohol use and 1.91 in a ≥1 drink per day group as a reference for non-drinkers. Indeed, alcohol intake is likely to have a deleterious effect for the development of diverticula, but no details on the amount of alcohol consumed were available for type of diverticula in their study. As we asked a detailed question with regard to type, times per week, and amount of alcohol, we were able to assess the precise consumption, which is a strength of this study. The biologic mechanisms linking alcohol to diverticulosis are unclear, but may involve colonic motility [18,19]. Berenson et al. [18] reported that intravenous administration of alcohol consistently decreases recto-sigmoid motor activity and correlates inversely with blood alcohol levels in humans. Wang et al. [19] demonstrated that alcohol inhibits colonic motility mainly through activation of NF-kB, subsequent upregulation of iNOS expression, and the increase of NO release in myenteric plexus in a rat model.

Smoking and the amount of pack-years was also found in this study to be another lifestyle risk factor for colonic diverticula and specifically the right-sided type. Only few data are available on the relationship between uncomplicated colonic diverticula and smoking [10,13]. Song et al. [10] found that smokers were 30% more likely to develop diverticulosis than nonsmokers after adjustment for important confounders, but this relationship was not statistically significant. Perry et al. [13] assessed smoking history defined as the total number of years smoked and found that patients with diverticulosis had longer tobacco use than those without. Possible mechanisms for the development of diverticulosis may include colonic microflora and colonic motility. Recently, colonic microflora has been shown to play an important role in the development and progression of diverticular disease [20]. Nicotine is known to inhibit the synthesis of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) [21], which may alter microflora. Furthermore, previous studies have shown that smoking increases chemical mediators such as vasoactive intestinal polypeptide (VIP) [22] and nitric oxide [23]. Milner et al. [24] revealed that the VIP content of the mucosa and whole wall was increased in diverticular disease. While, Tomita et al [25] reported that the colonic tissue of the diverticular-bearing segments is more strongly innervated by cholinergic nerves than normal segments of the colon. These findings suggest that chemical mediators affect colonic motility and intracolonic pressure, thereby possibly enhancing bulging of the colonic mucosa.

A limitation of this study is that several pathogenic factors reported to be associated with colonic diverticulosis were not included in the analysis; in particular, physical activity, familial and hereditary factors, obesity, and a detailed quantitative dietary history with regard to fiber and fat intake [3,4,12]. The absence of these factors could have confounded the relationships between alcohol and smoking. Although we demonstrated that the comorbidity of atherosclerotic vascular disease is associated with colonic diverticula on univariate analysis, this factor is not a true risk factor. We believe that patients with atherosclerotic vascular disease and colonic diverticula have common predisposing factors such as a low-fiber or high-fat diet and low physical activity.

In summary, our study shows that the overall prevalence of colonic diverticulosis was 25%, with 50% of cases on the right side. In addition to age, the amount of alcohol consumption and smoking were found to be identifiable risk factors for the development of uncomplicated colonic diverticulosis. These factors were also associated with right-sided and bilateral diverticula. Patients with atherosclerotic vascular disease are predisposed to colonic diverticula due to similar risk factors. Further study is needed to explore these associations as well as new risk factors from eastern and western countries.

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Author Contributions

Conceived and designed the experiments: NN TT JA MY MM NU TS. Performed the experiments: NN RN TA YK KS ST KW. Analyzed the data: TS. Contributed reagents/materials/analysis tools: RN TA YK KS ST KW NN. Wrote the paper: NN TI NU.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Ex vivo induction of IFN-λ3 by a TLR7 agonist determines response to Peg-IFN/Ribavirin therapy in chronic hepatitis C patients

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Abstract

Background Genetic variation around interleukin-28B (*IL28B*), encoding IFN-λ3, predict non-responders to pegylated interferon-α/ribavirin (Peg-IFN/RBV) therapy in chronic hepatitis C (CHC). However, it remains unclear the expression and the role of *IL28B* itself. The aim of this study is to develop easy and useful methods for the prediction of treatment outcomes.

Methods The mRNA and protein levels of IFN-λ3 induced by ex vivo stimulation of peripheral blood mononuclear cells (PBMC) or magnetically selected dendritic cells (DCs) with toll-like receptor agonists (TLR3; poly I:C, TLR7; R-837) were measured by the quantitative real-time polymerase chain reaction and our newly developed chemiluminescence enzyme immunoassays, respectively, and compared with the clinical data.

Results We found that BDCA-4$^+$ plasmacytoid and BDCA-3$^+$ myeloid DCs were the main producers of IFN-λs when stimulated with R-837 and poly I:C, respectively. Detectable levels of IFN-λs were inducible even in a small amount of PBMC, and IFN-λ3 was more robustly up-regulated by R-837 in PBMC of CHC patients with favorable genotype for the response to Peg-IFN/RBV (TT in *rs8099917*) than those with TG/GG. Importantly, the protein levels of IFN-λ3 induced by R-837 clearly differentiated the response to Peg-IFN/RBV treatment ($p = 1.0 \times 10^{-10}$), including cases that *IL28B* genotyping failed to predict the treatment response. The measurement of IFN-λ3 protein more accurately predicted treatment efficacies (95.7 %) than that of *IL28B* genotyping (65.2 %).

Conclusions Genetic variations around *IL28B* basically affect IFN-λ3 production, but different amounts of IFN-λ3 protein determines the outcomes of Peg-IFN/RBV treatment. This study, for the first time, presents compelling evidence that *IL28B* confer a functional phenotype.

Keywords Chronic hepatitis C · IL28B · IFN-λ3 · Peg-IFN/RBV

Abbreviations

ARFI	Acoustic radiation force impulses
CHC	Chronic hepatitis C
GWAS	Genome-wide association study
IL28B	Interleukin-28B
Peg-IFN/RBV	Pegylated interferon-α/ribavirin
PBMC	Peripheral blood mononuclear cells
SNP	Single nucleotide polymorphisms
SVR	Sustained viral response
TLR	Toll-like receptor
TVR	Transient viral response
VR	Viral response

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Introduction

Recently, we and others independently identified single nucleotide polymorphisms (SNPs) on chromosome 19 associated with the interleukin-28B gene (*IL28B*), encoding IFN-λ3, that were strongly associated with the response to pegylated interferon-α/ribavirin (Peg-IFN/RBV) in chronic hepatitis C (CHC) patients, through a genome-wide association study (GWAS) [1–3]. According to our results, about 80 % of CHC patients with the TT genotype (*rs8099917*) showed viral virologic response (VR), including SVR (sustained virologic response) or TVR (transient virologic response), whereas only about 20 % of HCV patients with the TG/GG genotype showed VR [1]. Thus, by genotyping of *IL28B*, we can predict the efficacy of Peg-IFN/RBV before beginning treatment, avoiding unnecessary side effects and the high cost of Peg-IFN/RBV treatment. However, it is still unknown whether genetic variation of *IL28B* is a functional phenotype for Peg-IFN/RBV treatment. In addition, genotyping of *IL28B* alone failed to predict about 20 % of the response [1], which would be reasonable because final products of the genes are affected by DNA methylation or chromatin modifications as well as genetic variations [4].

Type III IFNs, consisting of IFN-λ1, λ2, and λ3 (also known as *IL29*, *IL28A* and *IL28B*, respectively), have recently been characterized [5, 6]. IFN-λs up-regulate IFN-stimulated genes (ISGs) via Janus kinase/signal transducer and activator of transcription (Jak/STAT) intracellular signaling, inhibiting hepatitis B virus (HBV) or hepatitis C virus (HCV) replication [7]. Antiviral responses evoked by toll-like receptor (TLR)3 or TLR9 agonists are attenuated in IL28RA$^{-/-}$ mice [8], indicating the central role of IFN-λs in antiviral protection. Clinically, early virologic response by Peg-IFN/RBV is associated with a high probability of SVR in HCV patients [9]. Genetic variations of *IL28B* influence spontaneous clearance of HCV [10], or on-treatment viral kinetics [11]. These results suggest a mechanistic link between innate immunity and genetic variations of *IL28B*.

To recognize viruses and trigger innate antiviral responses, mammals have 2 independent receptors, retinoic acid-induced gene-I (RIG-I)-like receptors (RLRs) and TLRs, distinct families of pattern recognition receptors that sense nucleic acids derived from viruses [12]. RIG-I is a double-stranded RNA-binding DExD/H box RNA helicase that is essential for initiating the intracellular response to RNA viral infection [13]. However, NS3/4A, the major serine protease expressed by HCV, disrupts the RIG-I pathway through proteolysis of essential signaling components of IFN regulatory factor 3 (IRF-3) activation [13, 14], reducing immune response. Alternatively, the TLR-families play an important role in innate immune responses in mammals [15]. Among them, TLR3 recognizes viral double-stranded RNA, whereas TLR7 recognize single-stranded RNA. Because some TLRs ligands induce IFN-λ in human macrophages [16], contributing to antiviral defense and HCV is a single-stranded RNA [8], we hypothesized that IFN-λ3 induced via the TLR pathway might contribute to early antiviral response against HCV, which could lead to accurate prediction of treatment efficacy. Therefore, we investigated IFN-λs production in peripheral blood mononuclear cells (PBMC) in healthy volunteers or CHC patients by ex vivo stimulation with TLR agonists, and analyzed whether this method could predict the responses to Peg-IFN/RBV treatment in clinical practice.

Patients, materials, and methods

Study population

Blood samples were obtained from 12 healthy volunteers and 100 consecutive Japanese outpatients with CHC (genotype 1b and high viral load) who visited our hospital between April 2011 and March 2012. The study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of our institutes (NCGM-G-001023-01). Written informed consent was obtained from all volunteers and patients. All subjects were negative for HBV and human immunodeficiency virus, and did not have hepatocellular carcinoma. IFN treatment was not being given to any patient at the time blood samples were taken. The subjects were all evaluated for SNP near *IL28B* (*rs8099917*, *rs12979860*) using the InvaderPlus assay (Invader Chemistry, Madison, WI, USA) as previously reported [17].

Definition of treatment responses

Non-virologic response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pre-treatment baseline value within the first 12 weeks, and detectable viremia 24 weeks after initiation of treatment. VR was defined as achieving SVR or TVR. SVR was defined as undetectable HCV RNA in the serum 6 months after the end of treatment, whereas TVR was defined as reappearance of HCV RNA in the serum during or after completion of treatment.

Preparation of PBMC and selection of plasmacytoid or myeloid dendritic cells (DCs)

Whole blood anti-coagulated with EDTA was obtained from healthy volunteers and CHC patients. PBMC were isolated by Ficoll-Hypaque (Mediatech, Herndon, VA, USA) density

gradient centrifugation. BDCA-1, 3, 4^+DCs were negatively or positively selected by BDCA-1^+DC isolation kit, BDCA-3 MicroBead kit and BDCA-4/Neuropilin-1 MicroBead kit, respectively (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions.

Ex vivo induction of IFN-λ1, IFN-λ2, and IFN-λ3

After pre-treatment with or without 100 U/ml of IFN-α (Hayashibara Co. Ltd., Okayama, Japan) in 200 μl of Roswell Park Memorial Institutes (RPMI) medium supplemented with 10 % fetal bovine serum for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (TLR3 agonist; Imgenex, San Diego, CA, USA), or 5 μg/ml of imiquimod (R-837; TLR7 agonist, Imgenex) as previously reported [16]. For chemiluminescence enzyme immunoassays (CLEIA), 200,000 cells were subjected to the same stimulation protocol.

RNA isolation and cDNA synthesis

After stimulation with TRL-agonists for 4 h, the PBMC were lysed with ISOGEN-II (Nippon Gene, Tokyo, Japan). In some experiments, PBMC were harvested at each indicated time point. The lysate was supplemented with chloroform, incubated for 15 min on ice, and centrifuged at 22,000 g for 15 min. The aqueous layer was removed and precipitated with isopropanol. The RNA was pelleted by centrifugation, washed with ethanol, and dissolved in 20 μl of water. Reverse transcription was performed using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA).

Real-Time quantitative polymerase chain reaction (PCR)

Quantitative real-time PCR was performed to estimate IFN-λ1, IFN-λ2, and IFN-λ3 mRNA expression based on SYBR green fluorescence (Roche Diagnostics Japan), using TaqMan Universal PCR master mix (Roche Diagnostics Japan), according to the manufacturer's protocol. Relative gene expression was calculated as a fold induction. Data were analyzed using the $2\text{-}\Delta\Delta C(t)$ method with Sequence Detector version 1.7 software (Applied Biosystems, Carlsbad, CA, USA) and were normalized using human hypoxanthine phosphoribosyltransferase (HPRT). A standard curve was prepared by serial 10-fold dilutions of human cDNA. The curve was linear over 7 log units with a 0.998 correlation coefficient. Quantitative mRNA expression was determined by triplicate real-time PCR.

Chemiluminescence enzyme immunoassays

We recently developed a CLEIA system for IFN-λ3 that showed a wide detection range of 0.1–10,000 pg/ml with little or no cross-reactivity to IFN-λ1 or IFN-λ2 [18]. In addition, this CLEIA system can correctly detect IFN-λ3 from different *IL28B* genotypes.

Acoustic radiation force impulse (ARFI) elastography

For non-invasive evaluation of liver fibrosis, ARFI elastography was performed using a Siemens Acuson S2000™ ultrasound system (Mochida Siemens Medical System Co, Ltd, Tokyo, Japan) as previously reported [19]. We performed 5 measurements for each patient, and a median value was calculated. Liver stiffness was expressed as the shear wave velocity (m/s) and has been reported to be well correlated with histological liver fibrosis [19].

Statistical analyses

Continuous variables between groups were compared using the Mann–Whitney *U* test, and categorical data were compared using the Chi square test or Fisher's exact test. Correlations between continuous variables were searched using the Pearson correlation test. Values of $p < 0.05$ were considered significant.

Results

Genetic variation in *IL28B*

In CHC patients ($n = 100$), only 1 patient showed discrepancy between *rs8099917* and *rs12979860* with the same prediction for the treatment response by genotyping (TG in *rs8099917*, and TT in *rs12979860*). In addition, we recently reported that *rs8099917* has the greatest accuracy in determining the outcome of Peg-IFN/RBV treatment in Japanese patients [17]. Therefore, *rs8099917* is used in the following analyses. The major homologous (TT) in *rs8099917* is considered a predictive factor for a favorable response to Peg-IFN/RBV treatment, while having minor alleles (TG or GG) is considered predictive for non-responders. Seven of 12 healthy volunteers had the TT genotype of *IL28B* and 5 had TG genotype. In CHC patients, 59 patients had the TT genotype, 36 had TG, and 5 had GG in *rs8099917*.

BDCA-4^+ plasmacytoid DCs are the major producers of IFN-λs in response to R-837

Since Lauterbach et al. [20] found that human DCs expressing BDCA3 (CD141) in myeloid DC subsets were the primary producers of IFN-λs, we sought which cell types are the main producers of IFN-λs when stimulated with R-837. Because DCs from the different *IL28B*

genotype are supposed to produce different amounts of IFN-λs, we used DCs from healthy volunteers with TT genotype. After negative or positive magnetic selection of BDCA-1, 3, 4^+DCs using 100 ml of peripheral blood, each collection was stimulated with IFN-α, following poly I:C or R-837 as previously reported [16], and evaluated the mRNA of IFN-λs or the protein levels of IFN-λ3. We confirmed that BDCA-3^+DCs were the main producers of IFN-λs when stimulated with poly I:C as previously reported (Fig. 1a) [20]. Interestingly, when stimulated with R-837, positive selection of BDCA-4^+DCs (plasmacytoid DCs), not BDCA-3^+DCs, produced IFN-λs whereas depletion of BDCA-4^+DCs showed marked reduction of IFN-λs (Fig. 1b). Therefore, BDCA-4^+DCs were the main producers of IFN-λs when stimulated with R-837. Thus, different stimulation targeted different DC subsets to induce IFN-λs.

Induction of IFN-λs (IFN-λ1, IFN-λ2, and IFN-λ3) in PBMC from healthy volunteers

We confirmed that the main producers of IFN-λs were DCs. However, analyses of IFN-λs using DC subsets need a lot of blood, which cannot apply to patients, because

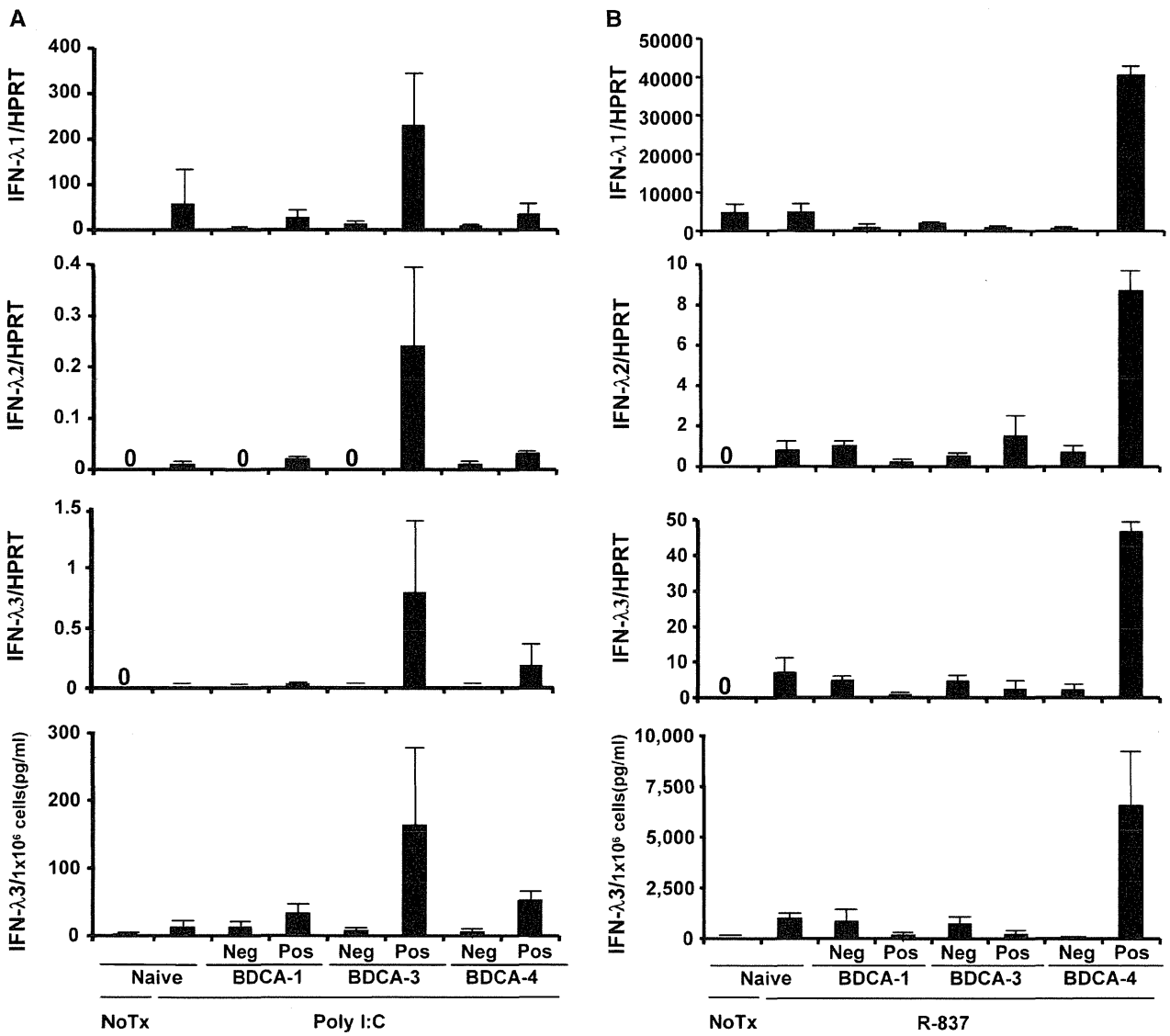

Fig. 1 IFN-λs were produced from different subsets of dendritic cells (DCs) when stimulated with different TLR agonists. BDCA-3^+ or BDCA-4^+DCs was negatively or positively selected using peripheral blood mononuclear cells (PBMC) from healthy volunteers ($n = 5$). PBMC or DCs were stimulated with IFN-α, following poly I:C (**a**) or R-837 (**b**). The mRNA and the protein levels of IFN-λs were determined by real-time PCR and CLEIA, respectively. *Neg* negative selection, *Pos* positive selection of each DCs

BDCA-3$^+$ or 4$^+$DCs are very minor subsets in peripheral blood (0.03, 0.5 %, respectively) [21]. Therefore, we examined if a small amount of PBMC, using 2–3 ml of whole blood from healthy volunteers with negative anti-HCV (Supplementary Table 1), still induced detectable levels of IFN-λs. We confirmed that, even with a small amount of PBMC, detectable levels of IFN-λs were induced by R-837 and the levels of IFN-λ3 were different between *IL28B* genotypes (Fig. 2a, b). Therefore, whole PBMC from healthy volunteers with the TT genotype were

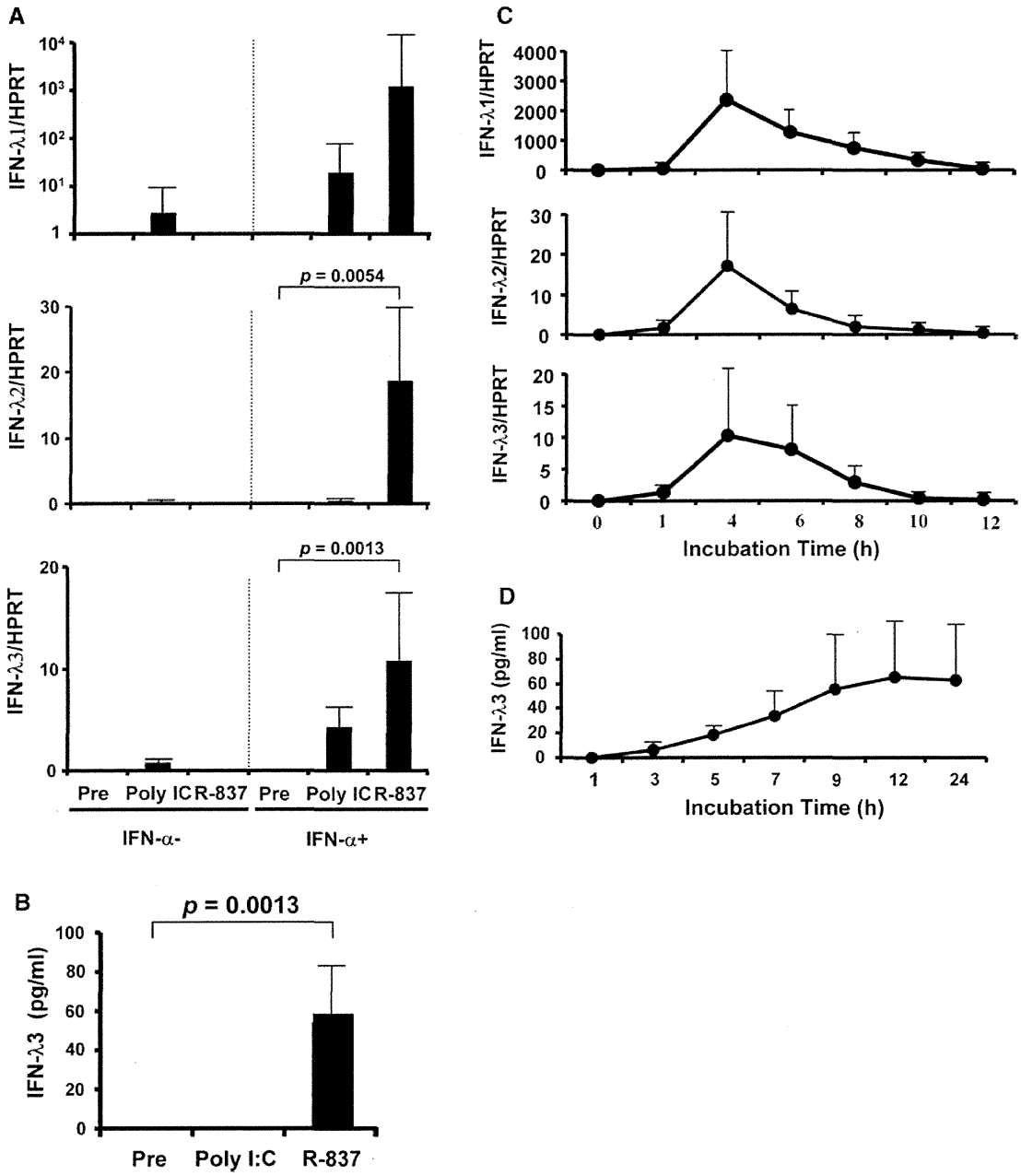

Fig. 2 Ex vivo induction of IFN-λs in PBMC from healthy volunteers. **a** mRNA expression levels of IFN-λs by real-time quantitative PCR. After pre-treatment with or without 100 U/ml of IFN-α for 16 h, 100,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist). After stimulation with TRL-agonists for 4 h, the PBMC were harvested. **b** Protein levels of IFN-λs. After pre-treatment with 100 U/ml of IFN-α, 200,000 of mononuclear cells were stimulated with 30 μg/ml of poly I:C (a TLR3 agonist) or 5 μg/ml of R-837 (a TLR7 agonist), and the supernatant was harvested 24 h after stimulation with TLR agonists. **c** Kinetics of IFN-λs mRNA levels. After pre-incubation with IFN-α for 16 h, PBMC was stimulated with 5 μg/ml of R-837. Real-time quantitative PCR was conducted at each time point. **d** CLEIA results for IFN-λ3 protein in the supernatant at each time point

used for the initial experiments (Fig. 2a–d). IFN-α or TLR-agonists alone failed to induce significant amounts of IFN-λs. All IFN-λs mRNA were strongly induced by R-837 along with IFN-α (Fig. 2a). Poly I:C also induced detectable levels of IFN-λs, but those were not prominent in our setting. Protein levels of IFN-λ3 were detectable only by R-837 (Fig. 2b). Therefore, we focused on R-837 in the following experiments. Next, we sought to confirm the kinetics of IFN-λs after stimulation with R-837. When PBMC from volunteers with the TT genotype ($n = 7$) were stimulated with R-837, an IFN-λ mRNA peak was observed 4 h after incubation, rapidly decreasing to undetectable levels at 12 h (Fig. 2c). In the CLEIA results, IFN-λ3 was detected in the supernatant beginning 3 h after stimulation with R-837, peaking at 9–12 h, then plateauing (Fig. 2d). From these observations, mRNA levels of IFN-λs and protein levels of IFN-λ3 induced by R-837 were measured at 4 and 24 h after R-837, respectively.

Patients' characteristics

Consecutive 100 HCV-RNA positive CHC patients with genotype 1b (35 male, 65 female) were enrolled in this study (Supplementary Table 2). Our patients with the TG/GG genotype were more prevalent (41.0 %, 41/100) than in the normal population (about 20 %), which was expected given that patients who had previously failed to benefit from HCV treatment tended to visit our center. Each patient who had histories of Peg-IFN/RBV ($n = 38$) was treated with Peg-IFN-α2b (1.5 μg per kg body weight (μg/kg) subcutaneously once a week) or PEG-IFN- α2a (180 μg once a week) plus RBV (600–1,000 mg daily depending on body weight) (Fig. 3). Since a reduction in the dose of PEG-IFN-α and RBV can contribute to less SVR [22], 2 patients with an adherence of <80 % dose for either drugs during the first 12 weeks who had shown NVR were excluded from "the known treatment-response" group. Patients who had been treated with IFN monotherapy ($n = 5$) were also excluded because *IL28B* was identified in patients having had Peg-IFN/RBV treatment, but not IFN monotherapy, through the GWAS. Among them ($n = 36$), 19 had shown TVR whereas 17 had shown NVR. After enrollment to this study, 10 treatment-naïve patients started Peg-IFN/RBV therapy. One patient showed undetectable HCV-RNA during therapy, and the therapy is ongoing (this patient was categorized in VR and/or TVR). Seven patients achieved SVR (categorized in VR and/or SVR), whereas 2 patients showed NVR (categorized in NVR). Therefore, 47 CHC patients were treatment-naïve and 46 CHC patients had the known response to Peg-IFN/RBV treatment. There were no differences in the characteristic backgrounds between patients with the TT and TG/GG genotypes in treatment-naïve CHC patients ($n = 47$) except for their serum γ-GTP level (32 ± 19 and 59 ± 53, respectively, $p = 0.017$) (Table 1), which were consistent with our recent report [23].

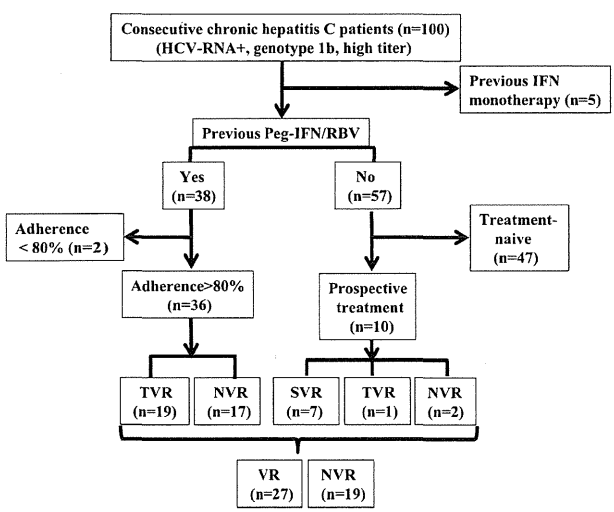

Fig. 3 Enrolled chronic hepatitis C patients with or without histories of treatment against HCV

Association of IFN-λ3 induction with genetic variations around *IL28B* in healthy volunteers or treatment-naïve CHC patients

R-837 induced higher levels of IFN-λ2 or IFN-λ3 mRNA in healthy volunteers with the TT genotype than in those with the TG/GG genotype whereas no differences were observed in IFN-λ1 (Fig. 4a). However, no statistical differences were observed in the protein levels of IFN-λ3

Table 1 Patients' characteristics of HCV treatment-naïve patients in TT or TG/GG genotype ($n = 47$)

	TT ($n = 28$)	TG/GG ($n = 19$)	p value
Age	66 ± 9	62 ± 14	ns
M:F	12:16	6:13	ns
WBC	4,493 ± 1,240	4,337 ± 1,096	ns
Hb	13.7 ± 1.7	13.7 ± 1.3	ns
Plt	15.5 ± 5.5	16.8 ± 6.0	ns
TP	7.7 ± 0.5	7.8 ± 0.5	ns
Alb	4.2 ± 0.5	4.4 ± 0.4	ns
AST	52 ± 30	44 ± 22	ns
ALT	56 ± 35	49 ± 29	ns
γ-GTP	32 ± 19	59 ± 53	0.017
ChE	280 ± 100	315 ± 87	ns
T-cho	170 ± 26	177 ± 37	ns
LDL	89 ± 23	97 ± 31	ns
HCV RNA	6.5 ± 0.6	6.3 ± 0.8	ns
ARFI[a]	1.50 ± 0.51	1.33 ± 0.37	ns

[a] ARFI (acoustic radiation force impulse) represents shear wave velocity (m/s)

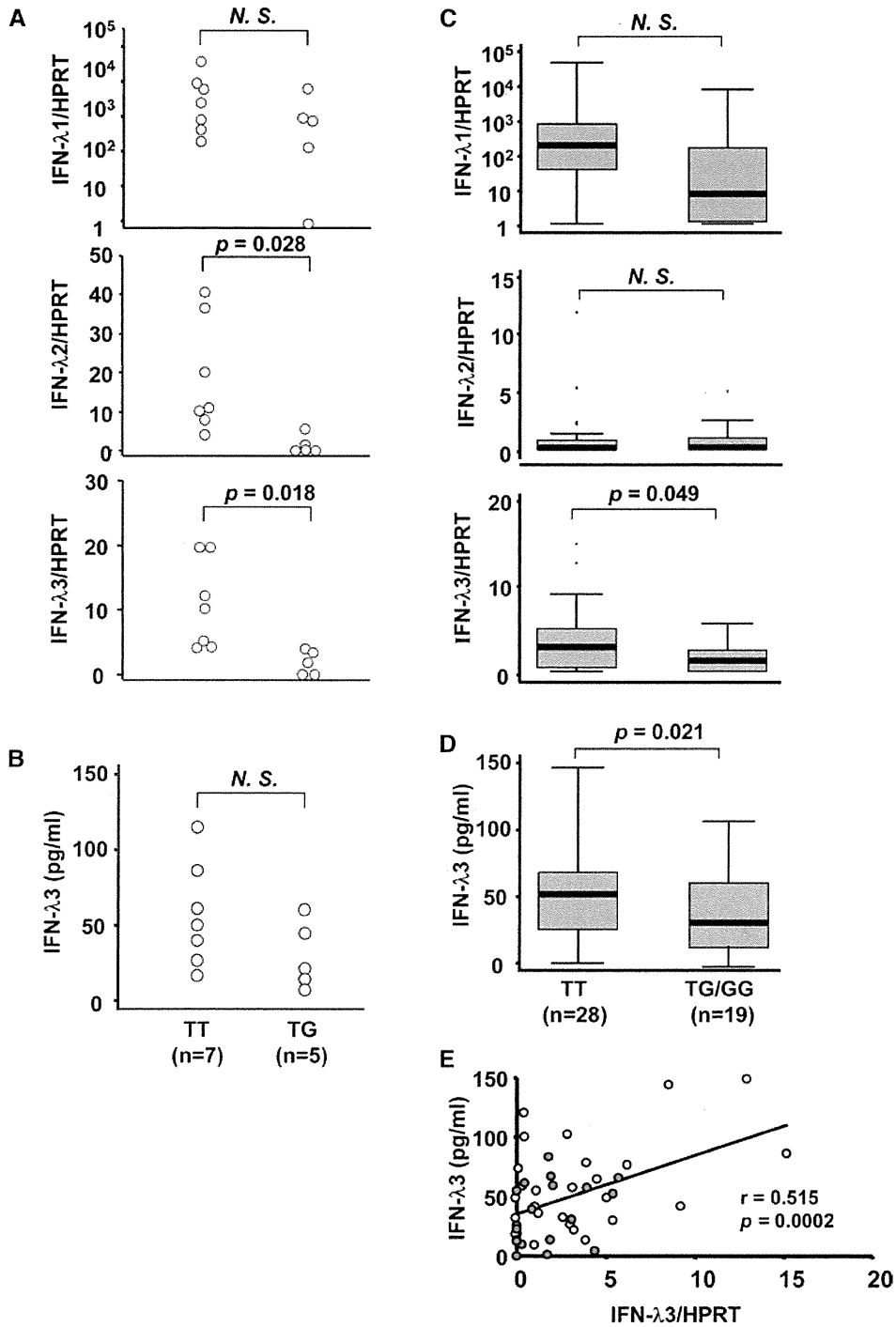

Fig. 4 Ex vivo induction of IFN-λs in PBMC from healthy volunteers and treatment-naïve CHC patients. **a** Differences of IFN-λs mRNA levels between each *IL28B* genotype in healthy volunteers ($n = 12$). After pre-treatment with 100 U/ml of IFN-α for 16 h, 100,000 of mononuclear cells were stimulated with 5 μg/ml of R-837. After stimulation with R-837 for 4 h, the PBMC were harvested. **b** Differences of IFN-λ3 protein levels between each IL28B genotype in healthy volunteers ($n = 12$) with or without R-837. **c** Differences of IFN-λs mRNA levels between each *IL28B* genotype in treatment-naïve CHC patients ($n = 47$). **d** Differences of IFN-λ3 protein levels between each *IL28B* genotypes in treatment-naïve CHC patients ($n = 47$) with or without R-837. **e** Correlation between mRNA and protein levels of IFN-λ3 in treatment-naïve CHC patients ($n = 47$). Each *open circle* represents TT genotype whereas each *closed circle* represents TG/GG genotype

between TT and TG genotype. The protein levels of IFN-λ3 were robustly induced by R-837 (Fig. 4b). These findings were similarly observed when using PBMC from treatment-naïve CHC patients (Fig. 4c, d). The protein levels of IFN-λ3 in PBMC from treatment-naïve CHC patients with TT genotype were significantly higher than those with TG/GG genotype. The IFN-λ3 mRNA levels were well correlated with those protein levels (Fig. 4e). Similar findings were obtained in all CHC patients ($n = 100$) (Supplementary Fig. 1).

Predictiveness of IFN-λ3 induction for response to Peg-IFN/RBV treatment

Among 46 HCV-RNA positive patients who had the known response to Peg-IFN/RBV, 27 patients showed VR and 19

Table 2 Patients' characteristics categorized in the response to treatment ($n = 46$)

	VR ($n = 27$)	NVR ($n = 19$)	p value
Age	63 ± 9	63 ± 9	ns
M:F	9:18	5:14	ns
TT:TG:GG	20:7:0	9:9:1	ns
WBC	4,181 ± 1,299	3,947 ± 1,127	ns
Hb	12.5 ± 2.0	13.1 ± 1.7	ns
Plt	18.1 ± 5.9	13.0 ± 3.6	0.002
TP	7.7 ± 0.6	7.8 ± 0.4	ns
Alb	4.4 ± 0.4	4.2 ± 0.3	ns
AST	46 ± 42	61 ± 35	ns
ALT	44 ± 44	73 ± 58	ns
γ-GTP	28 ± 22	51 ± 32	ns
ChE	293 ± 76	271 ± 75	ns
T-cho	178 ± 37	159 ± 23	ns
LDL	95 ± 22	88 ± 19	ns
HCV RNA	6.3 ± 0.6	6.3 ± 1.0	ns
Core 70 (W:M)	18:6	8:9	ns
ISDR (0:>0)	12:12	8:9	ns
ARFI[a]	1.20 ± 0.26	1.72 ± 0.34	<0.001

[a] ARFI (acoustic radiation force impulse) represents shear wave velocity (m/s)

showed NVR. Lower platelet counts and higher shear wave velocity were significantly observed in the NVR group (Table 2). The mRNA levels of IFN-λ3 were significantly higher in patients with VR than in those with NVR (Fig. 5c, $p = 0.0002$). The expression levels of IFN-λ1 (Fig. 5a) and IFN-λ2 (Fig. 5b) were also significantly higher in patients with VR although the statistical differences were markedly bigger than IFN-λ3. The protein levels of IFN-λ3 confirmed these results and more clearly differentiated between VR and NVR in patients with previous therapy ($n = 36$), in treatment-naïve patients with prospective therapy ($n = 10$), or combined ($n = 46$) (Fig. 5d, $p = 6.9 \times 10^{-9}$, $p = 0.014$, $p = 1.0 \times 10^{-10}$, respectively). Interestingly, 7 patients with the TG/GG genotype who showed VR demonstrated high IFN-λ3, whereas 8 patients with the TT genotype who showed NVR demonstrated low IFN-λ3 induction. Taken together, the response to Peg-IFN/RBV was mainly dependent on the capacity of IFN-λ3 production in the PBMC rather than on genetic variations in *IL28B*. Our method more accurately predicted treatment efficacies (44/46, 95.7 %) compared to *IL28B* genotyping (30/46, 65.2 %) when cut-off of IFN-λ3 protein levels was set at the median value (47.6 pg/ml) (Table 3).

Discussion

The present study demonstrated that the amount of endogenous IFN-λ3 in PBMC induced by the TLR7 agonist determines the outcome of Peg-IFN/RBV therapy, though its induction was basically dependent on the *IL28B* genotype. Since this method can evaluate the final gene products, the genetic factor or epigenetic factor is not necessary for consideration, which could provide more accurate prediction of the response to Peg-IFN/RBV therapy than *IL28B* genotyping. In addition, an annoying informed consent about gene handling is not necessary, and the cost is lower than genomic analyses. IFN-based treatment with novel drugs such as protease inhibitors or polymerase inhibitors could be still predictive by this method because genetic variations in *IL28B* are strongly associated with response to telaprevir/Peg-IFN/RBV treatment [24].

IFN-λs were shown to inhibit the replication of a number of viruses in vitro, including HBV or HCV [5–7], by up-regulation of ISGs. Several GWAS studies suggested that IFN-λ3 played a key role in the response to HCV [1–3, 10, 11]. Meanwhile, type III IFNs, produced by hepatocytes, in response to HCV infection, predominantly lead functional ISGs induction in comparison to type I IFNs [25]. The combination of *IL28B* genotype between recipients and donors determines the outcome of Peg-IFN/RBV therapy for recurrent hepatitis C after liver transplantation [26]. Therefore, a favorable response to anti-HCV treatment might be dependent on the amount of endogenous IFN-λ3 produced by both lymphocytes and hepatocytes.

Unsuccessful induction of IFN-λs by IFN-α or TLR-agonists alone (Fig. 1a) may explain controversial results of IFN-λ3 expression in either PBMC or intrahepatic lymphocytes from pre-treatment patients [1, 3, 23, 27]. IFN-α up-regulates the expression of TLR, TRIF, and MyD88, common adaptor molecules associated with TLR signaling [16]. UV-inactivated viruses, as well as infectious viruses, can induce IFN-λ, suggesting viruses can be sensed through a non-infectious route such as endocytosis of infectious or non-infectious viral particles [28]. TLR7 appears to play an important role in the induction of antiviral responses against single-stranded RNA viruses [29–31], and imidazoquinoline activates immune cells via the TLR7-MyD88-dependent signaling pathway [32]. Synthetic TLR7 agonists have recently been characterized with respect to their ability to induce cytokines [33]. Furthermore, in our study, IFN-λ3 induced by R-837 clearly differentiated the response to Peg-IFN/RBV treatment. Therefore, sequential stimulation with IFN-α following a TLR7 agonist (R-837) may mimic IFN therapy in CHC patients in terms of IFN-λs induction, and our sensitive CLEIA system may contribute to clear differentiation among the responses to Peg-IFN/RBV treatment in this study. It has recently been reported that expression levels and function of TLR7 were impaired in HCV-infected human hepatoma cells [34]. However, in the present study,

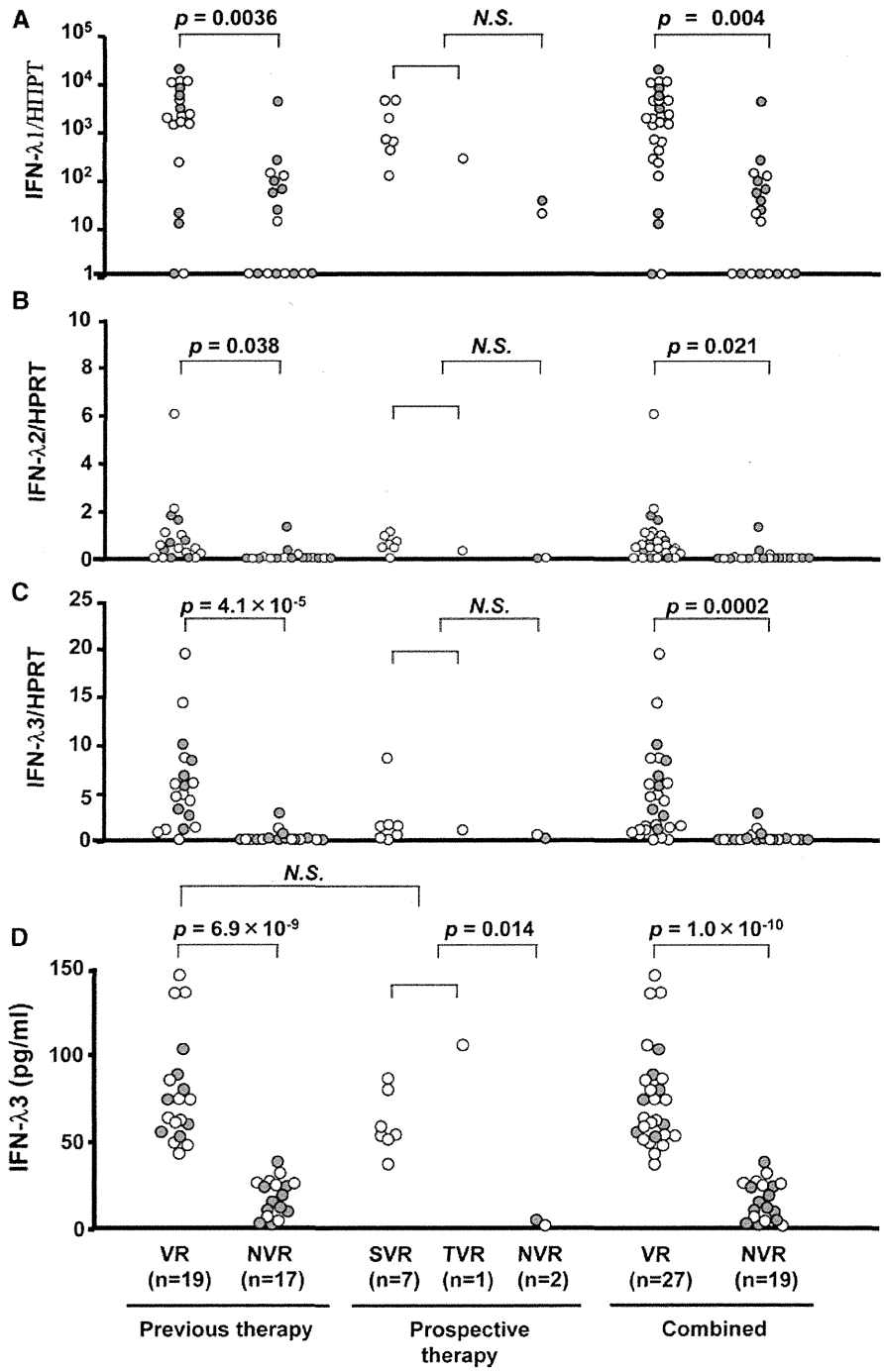

Fig. 5 The mRNA and protein levels of IFN-λ3 ex vivo induced by R-837 and treatment responses in CHC patients with "the known response to Peg-IFN/RBV". The mRNA levels of IFN-λ1 (**a**), IFN-λ2 (**b**), and IFN-λ3 (**c**) of virologic responders (VR) ($n = 27$) and non-virologic responders (NVR) ($n = 19$) were shown. (**d**) Protein levels of IFN-λ3 in CHC patients who had previously failed Peg-IFN/RBV therapy (previous therapy, $n = 36$) and who prospectively treated with Peg-IFN/RBV (prospective therapy, $n = 10$). Combined figures were also shown ($n = 46$). Each *open circle* represents TT genotype whereas each *closed circle* represents TG/GG genotype

a TLR7 agonist was able to induce IFN-λs in PBMC from HCV patients, although the response was slightly impaired in HCV patients compared with that in healthy volunteers. Therefore, impairment of expression or function of TLR7 in HCV patients may not be a critical factor for whole innate immunity.

IFN-λs display high sequence homology. In particular, IFN-λ2 and IFN-λ3 are virtually identical with 96 % amino acid homology [6]. However, the GWAS revealed that only SNPs near the *IL28B*, not *IL28A* or *IL29*, showed strong associations with response to Peg-IFN/RBV treatment [1–3]. In the current study, we found that the expression level of IFN-λ3 in the PBMCs was better correlated with genetic variations in *IL28B* or response to Peg-IFN/RBV treatment. Furthermore, the antiviral effect of recombinant IFN-λ3 was more potent than those of IFN-λ1 or IFN-λ2 [35].

Table 3 Correct prediction rate by genotyping of *IL28B* or IFN-λ3 value (cut-off 47.6 pg/ml)

	IL28B genotype				IFN-λ3		
	TT	TG/GG			High	Low	
VR	**20**	7	27	VR	**25**	2	27
NVR	9	**10**	19	NVR	0	**19**	19
	29	17	46		25	21	46

Bold values indicated correct prediction in each category

Collectively, these findings may support our GWAS data. Alternatively, there are significant differences in IFN-λ2 as well as IFN-λ3 between genotypes or treatment response. The specific primers and probe sets for the IFN-lambda family that we previously developed achieved approximately 10^7-fold specificity in each IFN-λ [18]. However, Osterlund et al. [36] reported that both IFN-λ2 and IFN-λ3 were regulated by a similar pathway of IRF7 resembling those of IFN-α gene expression. Therefore, it is possible that the upregulation of IFN-λ3 could affect that of IFN-λ2 because the homology of regulatory sequence between IFN-λ2 and IFN-λ3 is pretty high. On the other hand, IFN-λ1 and λ2, λ3 show rather low homology (81 %), which could explain the different responses among them.

About 20 % of the HCV patients with the TT genotype failed to respond to the treatment [1]. Indeed, in the current study, 8 of 28 (28.6 %) patients with favorable *IL28B* genotype (TT) showed NVR in Peg-IFN/RBV therapy. Interestingly, all these cases who showed NVR despite their favorable *IL28B* genotype demonstrated low IFN-λ3 production (Fig. 5a–d). Generally, poor response to HCV treatment is related to a number of factors that are unlikely to be solely due to the *IL28B* genotype, such as greater age, male gender, viral factors and liver fibrosis [37, 38]. Therefore, we have tried to calculate multivariate logistic regression to find the most predictive factors affecting the treatment response including clinical backgrounds and the current data. However, the calculations were impossible because of "complete separation" between the treatment-response groups on ELISA data. Liver fibrosis may be attributed to hyporesponsiveness to Peg-IFN/RBV because surrogate markers of liver fibrosis (low platelet counts and high shear wave velocity) were significantly observed in our NVR cases (Table 2). However, both of surrogate markers showed substantial overlap between VR and NVR (Supplementary Fig. 2). Other factors including genetic deficiency of molecules in the TLR7 signaling pathway [39] may affect IFN-λs production. Meanwhile, 7 of 18 (38.9 %) patients with the unfavorable *IL28B* genotype (TG/GG) showed VR in Peg-IFN/RBV therapy (Table 3) and all of these VR patients showed high IFN-λ3 production. Because recombinant IFN-λ from any *IL28B* genetic variant similarly represses HCV RNA [40], the amount of IFN-λ, whatever the genetic variations of *IL28B* are, would be important for antiviral effects. Enhanced transcriptional regulations of *IL28B* could partially be attributed to high IFN-λ3 production in patients with TG/GG [41]. Alternatively, the number of BDCA-4$^+$DCs was well correlated with IFN-λ3 production when PBMC from healthy volunteers was stimulated with R-837 (Supplementary Fig. 3), which suggest that the number of BDCA-4$^+$DC strongly affect IFN-λ3 production in our setting. However, the precise mechanisms of regulating IFN-λ3 production or the number of BDCA-4$^+$DCs in CHC patients should be addressed in the future. Importantly, our methods clearly predict the effectiveness of Peg-IFN/RBV including these exceptional cases.

In the present study, neither the mRNA nor the protein levels of IFN-λ3 were much different among genetic variations in *IL28B* (Fig. 4c, d). It is possible that the exceptional cases of IFN-λ3 levels (e.g., low IFN-λ3 levels in the TT genotype and high levels in the TG/GG genotype) affected the statistical differences. Substantial overlap in the mRNA levels of IFN-λ3 was observed between the different treatment response groups whereas the protein levels of IFN-λ3 clearly differentiated (Fig. 5a–d). One possibility would be that our newly developed CLEIA system [18] is more sensitive than real-time PCR. Moreover, IFN-λs mRNA levels were rapidly induced by R-837, and these effects were limited to several hours after stimulation (Fig. 2c) whereas protein levels of IFN-λ3 were stable from 9 to 24 h after R-837 stimulation (Fig. 2d). In the prospective study, there were no statistical differences in the mRNA levels of IFN-λs. However, the increase of the number of these patients would clarify the differences because the trend of these levels in the prospective study was similar to those in the previously treated group.

In conclusion, our findings suggest that genetic variations in *IL28B* basically affect IFN-λ3 production; however, the amount of endogenous IFN-λ3 determines the outcome of Peg-IFN/RBV therapy. This study, for the first time, presents compelling evidence that genetic variations in *IL28B* confer a functional phenotype and potentially explains our GWAS data. In addition, these results may explain discrepant cases related to *IL28B* genotyping in the response to Peg-IFN/RBV treatment. Thus, ex vivo induction of IFN-λ3 in PBMC by a TLR7-agonist may be a more accurate predictive method for determining the outcome of Peg-IFN/RBV therapy.

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Conflict of interest Tatsuji Kimura is an employee of the Institute of Immunology Co., Ltd. All other authors have nothing to declare.

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