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Neutrophil–lymphocyte ratio reflects hepatocellular carcinoma recurrence after liver transplantation via inflammatory microenvironment

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Background & Aims: Although the Milan criteria (MC) have been used to select liver transplantation candidates among patients with hepatocellular carcinoma (HCC), many patients exceeding the MC have shown good prognosis. Preoperative neutrophil–lymphocyte ratio (NLR) is a predictor of patient prognosis, but its mechanism has never been clarified.

Methods: We assessed outcomes in 158 patients who had undergone living-donor liver transplantation (LDLT) for HCC. Recurrence-free survival (RFS) was determined in patients with high (≥4) and low (<4) NLR. Levels of expression of vascular endothelial growth factor (VEGF), interleukin (IL)-8, IL-17, CD68, and CD163 were measured.

Results: The 5-year RFS rate was significantly lower in patients with high (n = 26) than with low (n = 132) NLR (30.3% *vs.* 89.0%, $p < 0.0001$), in patients with high (n = 15) than with low (n = 79) NLR who met the MC (73.6% *vs.* 100%, $p = 0.0008$) and in patients with high (n = 11) than with low (n = 53) NLR who exceeded the MC (0% *vs.* 76.1%, $p = 0.0002$). Tumor expression of VEGF, IL8, IL-17, CD68, and CD163 was similar in the high and low NLR groups, but serum and peritumoral IL-17 levels were significantly higher in the high-NLR group ($p = 0.01$ each). The density of peritumoral CD163 correlated with the density of peritumoral IL-17-producing cells ($p = 0.04$) and was significantly higher in the high-NLR group ($p = 0.005$).

Conclusions: NLR predicts outcomes after LDLT for HCC via the inflammatory tumor microenvironment. Combined with the MC, NLR may be a new criterion for LDLT candidates with HCC.

Keywords: NLR; Liver transplantation; HCC; IL 17; TAM.
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Abbreviations: HCC, hepatocellular carcinoma; MC, Milan criteria; NLR, neutrophil–lymphocyte ratio; VEGF, vascular endothelial growth factor; LT, liver transplantation; LDLT, living donor liver transplantation; DDLT, deceased donor liver transplantation; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; OS, overall survival; RFS, recurrence-free survival; CRP, C-reactive protein; TAM, tumor-associated macrophage.

Introduction

Liver transplantation (LT) has been established as a standard therapy for patients with hepatocellular carcinoma (HCC) and end-stage liver diseases since the introduction of the Milan criteria (MC) in 1996. These criteria specify that LT should be performed in patients with a single tumor ≤5 cm in diameter, or ≤3 tumors, each ≤3 cm in diameter [1]. Despite excellent outcomes in patients meeting the MC, some experience tumor recurrence. In contrast, some patients exceeding the MC may have favorable outcomes [2], partly because the MC are based solely on preoperative diagnostic imaging, with no consideration of the tumor biological grade. Expanded criteria for the selection of LT candidates among patients with HCC have therefore been proposed [2–4].

Systemic inflammatory responses have been shown to reflect the promotion of angiogenesis, and DNA damage and tumor invasion through upregulation of cytokines [5–7]. A simple index of systemic inflammation is the neutrophil–lymphocyte ratio (NLR). Elevated NLR has recently been shown associated with poorer prognosis in patients with various types of malignant tumors, including colorectal cancer, HCC, intrahepatic cholangiocellular carcinoma, and pancreatic cancer [8–11]. Furthermore, elevated NLR have shown a significant correlation with poor outcome in patients undergoing LT for HCC [12]. One mechanism by which elevated NLR can lead to a higher tumor recurrence rate involves an increased number of circulating neutrophils secreting the vascular endothelial growth factor (VEGF), resulting in higher levels of VEGF in the tumors. None of these studies, however, have clarified the expression of VEGF and other tumor growth or angiogenic factors.

Living donor LT (LDLT) has become more widely used in Japan and other Asian countries than deceased donor LT (DDLT), which is more widely used in the United States. In contrast to DDLT, LDLT usually utilizes a blood-related donor graft, differs in graft

size, and involves a shorter waiting time for transplantation. LDLT has been shown to be superior to DDLT for HCC patients [13]. Therefore, the criteria for selecting candidates for LDLT and DDLT in patients with HCC differ.

To determine whether NLR can be used as a criterion for selecting HCC patients for LDLT, we have assessed the impact of elevated NLR on long-term outcomes in these patients and suggested its molecular mechanism.

Materials and methods

Patient selection and operative techniques

We enrolled 158 consecutive HCC patients who underwent LDLT at Kyushu University Hospital, Fukuoka, Japan, between July 1999 and March 2011. All patients provided full written informed consent, and the study was approved by the Ethical Committee of Kyushu University.

Preoperative tumor evaluation was done by diagnostic imaging methods, including abdominal ultrasonography, thoracic, and abdominal computed tomography (CT), hepatic angiography with CT, and magnetic resonance imaging. Patients who underwent pretreatment for HCC, including transcatheter arterial chemoembolization (TACE) were evaluated >3 months after treatment. Neutrophil and lymphocyte counts were routinely measured on the day before transplantation, with NLR calculated by dividing neutrophil count by lymphocyte count. For patients with multiple tumors, the main tumors were selected by an expert pathologist. LDLT procedures for both donors and recipients have been previously described [14]. Simultaneous splenectomy during LT was performed if recipients were positive for HCV or showed high portal venous pressure.

Immunosuppression and patient follow-up

Following LDLT, patients were treated with mycophenolate mofetil (Cellcept®; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) and injected steroids (1 g/day intraoperatively, tapered to zero by day 7), followed by maintenance with low-dose tacrolimus (Prograf®; Astellas, Tokyo, Japan) or cyclosporine (Neoral®; Novartis, Tokyo, Japan). Patients were followed-up monthly for the first 6 months, including assays of their peripheral blood for tumor markers such as AFP and ultrasonography, and enhanced CT every 6 months [4].

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from recipients' explanted liver tissues using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using a Superscript III Reverse Transcriptase Kit® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. qPCR was performed using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) PCR protocol, in which fluorescence emission was attributable to the binding of SYBR Green I dye to amplified products. The specific *VEGF* primer sequences were: sense, 5′-GGA GGG CAG AAT CAT CAC GAA-3′ and antisense, 5′-ATC GCA TCA GGG GCA CAC AG-3′; the interleukin *(IL)-8* primers were: sense, 5′-GAA GAG GGC TGA GAA TTC AT-3′ and antisense, 5′-AAT CTT GTA TTG CAT CTG GC-3′; and the *β-actin* primers were: sense, 5′-CTG GCA CCA CAC CTT CTA CAA TG-3′ and antisense, 5′-GGC GTA CAG GGA TAG CAC AGG-3′.

Immunohistochemistry

Explanted liver specimens were fixed in 10% buffered formalin, embedded in paraffin, pretreated in a microwave at 100 °C for 20 min, and incubated with primary antibodies to CD68 (KPI, 1:300, DAKO, Glostrup, Denmark) and CD163 (10D6, 1:200, Novocastra, Newcastle, UK). Immunohistochemical staining was detected by an Envision+ system and DAB kit (DAKO). For IL-17 immunohistochemical staining, sections were autoclaved at 121 °C for 20 min and incubated with primary antibody to IL-17 (goat monoclonal IgG, 1:200, R&D Systems, Minneapolis, MN, USA). Stained cells were counted in tumors and in peritumoral non-cancerous liver tissues.

Necrotic tumors were excluded from these assays and viable tumors were selected for staining.

Enzyme-linked immunosorbent assay (ELISA)

Whole blood samples from all enrolled patients were collected in the operating rooms before transplantation and centrifuged at 3000 rpm for 10 min, and serum samples immediately stored at −80 °C until use. Serum concentrations of VEGF, IL-8, and IL-17 were determined using the respective Quantikine® ELISA kits (R&D Systems), according to the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed using JMP® software (SAS Institute, Cary, NC, USA). Survival rates, including overall survival (OS) and recurrence-free survival (RFS), were calculated using the Kaplan–Meier method and evaluated with the log-rank test. Qualitative variables were compared using χ^2 tests, and quantitative variables were compared using Wilcoxon tests. Statistical significance was defined as $p < 0.05$.

Results

Patient background

The 158 patients who underwent LDLT for HCC at Kyushu University Hospital between July 1999 and March 2011 consisted of 92 males and 66 females. Their mean age was 57 years, 114 were infected with hepatitis C virus, and 94 met the MC. Of these 158 patients, 101 received pre-transplant treatment for HCC, including 32 who received percutaneous ethanol injection therapy, 26 who received microwave coagulation therapy, 58 who underwent radiofrequency ablation, 56 who received chemotherapy, 78 who underwent TACE and 9 who underwent hepatectomy. Specimens from 9 patients contained only necrotic tumors, 2 in the NLR ⩾4 group, and 7 in the NLR <4 group; these samples were excluded from immunohistochemistry and PCR assays.

The median follow-up period in the 158 patients was 40.3 months. Their 1-, 3-, and 5-year OS rates were 93.2%, 83.8%, and 80.3%, respectively, and their 1-, 3-, and 5-year RFS rates were 90.8%, 84.5%, and 82.7%, respectively. RFS was worse in patients who had received pre-transplant therapies compared to those who had not, with 1-, 3-, and 5-year RFS rates of 88.9% *vs.* 94.2%, 79.0% *vs.* 94.2%, and 76.8% *vs.* 94.2%, respectively ($p = 0.03$, data not shown).

Correlation between NLR and HCC recurrence following LDLT

To determine whether elevated NLR was correlated with HCC recurrence after LDLT, we performed Cox proportional hazard model analysis. We found that each integral increase in NLR was significantly associated with a hazard ratio (HR) of 1.2 for HCC recurrence (Supplementary Fig. 1A). Using NLR cut-offs of 1, 2, 3, 4, 5, and 6 and comparing RFS rates, we showed that all NLRs, except NLR ⩾1, were statistically correlated with HCC recurrence after LDLT by log-rank test (Table 1). Of these, an NLR of 4 was the most significant, with a Chi-square value of 15.2 and a p value <0.0001. We therefore utilized an NLR cut-off of 4 as a risk factor for HCC recurrence. RFS rates relative to NLRs of 2, 3, 5, and 6 are shown in Supplementary Fig. 1B–E.

Of the 158 patients, 26 (16.5%) had an NLR ⩾4. Clinical, surgical, and pathological data in the low (<4) NLR (n = 132) and high (⩾4) NLR (n = 26) groups are compared in Table 2. None of these factors differed significantly in the two groups, except for

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Table 1. Correlation between each NLR cut-off and HCC recurrence using the Kaplan–Meier method.

Cut-off value of NLR	1-yr RFS*	3-yr RFS*	5-yr RFS*	Chi-square	*p* value
NLR ≥6 (n = 11)	72.7% *vs.* 92.4%	42.4% *vs.* 87.6%	42.4% *vs.* 85.8%	11.7	0.0006
NLR ≥5 (n = 18)	83.3% *vs.* 92.0%	59.1% *vs.* 87.9%	59.1% *vs.* 86.0%	6.86	0.009
NLR ≥4 (n = 26)	78.8% *vs.* 93.2%	60.5% *vs.* 89.0%	30.3% *vs.* 89.0%	15.2	<0.0001
NLR ≥3 (n = 52)	81.4% *vs.* 95.7%	72.6% *vs.* 90.4%	66.0% *vs.* 90.4%	9.98	0.002
NLR ≥2 (n = 83)	84.4% *vs.* 98.5%	76.1% *vs.* 94.4%	72.5% *vs.* 94.4%	9.77	0.002
NLR ≥1 (n = 131)	90.5% *vs.* 95.2%	83.5% *vs.* 90.2%	81.4% *vs.* 90.2%	0.55	0.456

*RFS in the high *vs.* the low NLR group.
HCC, hepatocellular carcinoma; NLR, neutrophil–lymphocyte ratio; RFS, recurrence free survival.

Table 2. Demographic and clinical characteristics of patients in the low NLR and high NLR groups.

	NLR <4 (n = 132)	NLR ≥4 (n = 26)	*p* value
Patient background			
Recipient's age (yr), mean (min.-max.)	58 (21-68)	54 (40-73)	0.06
Recipient's sex (male/female), n	77/55	15/11	0.95
Recipient's BMI (kg/m²), mean ± SD	23.8 ± 0.3	24.0 ± 0.7	0.89
Etiology (HBV/HCV/NBNC), n	21/92/19	4/22/0	0.11
MELD score, mean ± SD	11.0 ± 0.4	12.1 ± 1.3	0.50
CRP (mg/dl), mean ± SD	0.50 ± 0.1	1.2 ± 0.2	<0.0001
Pretransplant therapy for HCC (yes/no), n	83/49	18/8	0.53
Operative factor			
Operative time (min), mean ± SD	860 ± 49	795 ± 110	0.84
Intraoperative bleeding (ml), mean ± SD	5060 ± 588	8315 ± 1352	0.21
Graft (LL/RL/PS/Dual), n	79/49/3/1	19/7/0/0	0.10
Immune suppression (FK506/CyA), n	53/79	16/10	0.04
Tumoral factor			
α-fetoprotein (ng/ml), mean (min.-max.)	446 (1.9-26,525)	3289 (1.6-43,107)	0.79
Des-gamma-carboxy prothrombin (mAU/ml), mean (min.-max.)	315 (3-5934)	879 (7-13,691)	0.63
Maximum tumor size (cm), mean ± SD	2.2 ± 0.1	2.4 ± 0.3	0.99
Number of tumors, n	3.8 ± 0.3	3.5 ± 0.7	0.69
Tumor differentiation (well/moderate/poor), n	14/83/35	4/13/9	0.47
Vascular invasion (yes/no), n	47/85	12/16	0.31

BMI, body mass index; CRP, C reactive protein; CyA, cyclosporine; FK506, tacrolimus; HBV, hepatitis B virus; HCV, hepatitis C virus; LL, left lobe; MELD, model for end-stage liver disease; NBNC, non-HBV, and non-HCV; PS, posterior segment; RL, right lobe.

immunosuppressive agents. Of the patients in the low-NLR group, 53 received FK506 and 79 received cyclosporine A; of the patients in the high-NLR group, 16 received FK506 and 10 received cyclosporine A ($p = 0.04$). We also observed a significant difference in the low and high NLR groups in pretransplant C-reactive protein (CRP) concentration (0.50 mg/dl *vs.* 1.2 mg/dl, $p < 0.0001$). NLR did not correlate with any tumor factor, including serum tumor markers, tumor number, size, or microvascular invasion.

When we compared survival outcomes in the two groups, we found that the 1-, 3-, and 5-year OS rates were significantly lower in the high (80.1%, 66.6%, and 57.1%, respectively) than in the low (95.9%, 88.4%, and 84.1%, respectively) NLR group ($p = 0.002$, Fig. 1A). We also found that NLR ⩾4 significantly correlated with HCC recurrence following LDLT, with 1-, 3-, and 5-year RFS rates being significantly lower in the high (78.8%, 60.5%, and 30.3%, respectively) than in the low (93.2%, 89.0%, and 89.0%, respectively) NLR group ($p < 0.0001$; Fig. 1B). Interestingly, all 12 patients in the high-NLR group who experienced recurrences did so within 3 years of LDLT, suggesting that NLR may be a marker of early HCC recurrence after LDLT.

To date, the MC has been the gold standard for selecting HCC patients as candidates for LT. To confirm whether NLR predicts the outcome of LDLT for HCC patients independent of MC, a multivariate analysis was performed. As shown in Table 3, NLR ⩾4 was an independent factor affecting HCC recurrence after LDLT, with a HR of 6.24 ($p = 0.0002$). In addition, we performed multivariate analysis including all tumor factors shown in Table 2, which still showed NLR ⩾4 was significant factors associated HCC recurrence after LDLT (Supplementary Table 1).

We also compared survival outcomes in patients with high and low NLR who did or did not meet the MC. Of the 94 recipients who met the MC, 15 had high NLR, with 1-, 3-, and 5-year RFS rates of 100%, 73.9%, and 73.9%, respectively; in contrast, none of the 79 recipients with low NLR showed HCC recurrence ($p = 0.0008$, Fig. 1C). Similarly, among the 64 recipients who did

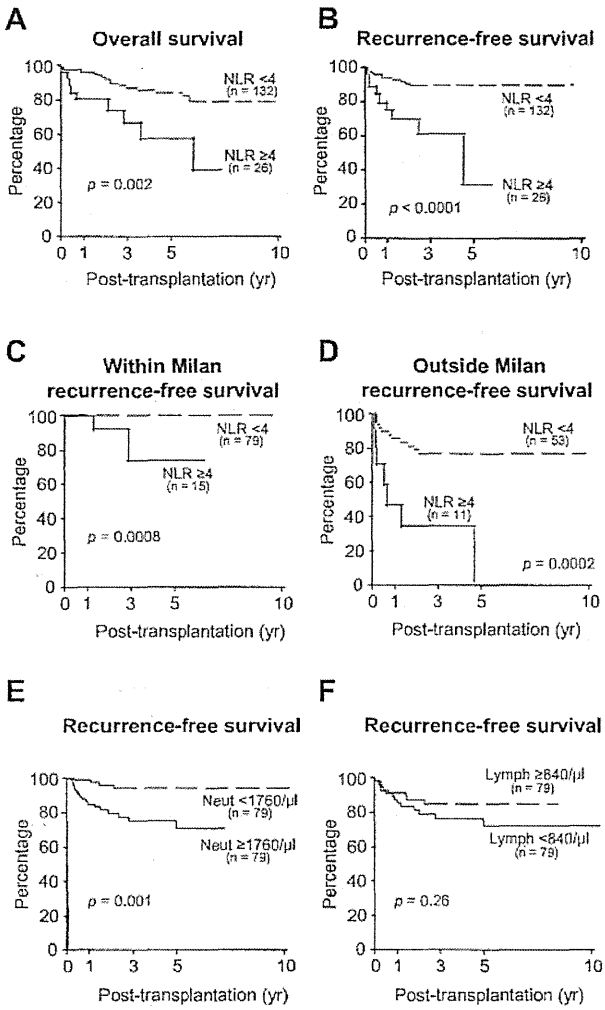

Fig. 1. Survival outcomes in patients with high (≥4) and low (<4) NLR. (A) OS rates and (B) RFS rates in the high- and low-NLR groups. (C and D) RFS rates in patients with high and low NLR who did (C) and did not (D) meet the MC. (E and F) RFS rates in patients above and below the (E) median neutrophil count (1760/μl) and the (F) median lymphocyte count (840/μl).

Table 3. Multivariate analysis of factors affecting HCC recurrence after liver transplantation.

Variables	HR	95% CI	*p* value
Milan Criteria	15.9	4.58-100	<0.0001
NLR ≥4	6.24	2.52-15.0	0.0002

HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; NLR, neutrophil-lymphocyte ratio.

not meet the MC, 11 with high NLR showed poorer survival outcomes than 53 with low NLR (Fig. 1D). The 1-, 3-, and 5-year RFS rates were 84.6%, 76.1%, and 76.1%, respectively, in the low-NLR group, and 46.7%, 35%, and 0%, respectively, in the high-NLR group ($p = 0.0008$). These results suggest that LT recipients with high NLR should be monitored carefully for HCC recurrence, even if they meet the MC, and that recipients with low NLR outside the MC may be feasible candidates for LDLT.

Correlation between neutrophil rather than lymphocyte counts and HCC recurrence

We also compared survival outcomes relative to neutrophil and lymphocyte counts. The 158 LT recipients were divided into two groups according to the median neutrophil count, those with high (≥1760/μl, n = 79) and low (<1760/μl, n = 79) neutrophil groups. The 1-, 3-, and 5-year RFS rates were 97.1%, 93.6%, and 93.6%, respectively, in the low-neutrophil group, and 84.3%, 74.9%, and 70.5%, respectively, in the high-neutrophil group ($p = 0.001$, Fig. 1E). In contrast, when we divided the 158 recipients according to the median lymphocyte count, we found that the 1-, 3-, and 5-year RFS rates were 92.9%, 87.6%, and 87.6%, respectively, in the high-lymphocyte group (≥840/μl, n = 79), and 88.8%, 81.2%, and 77.7%, respectively, in the low-lymphocyte group (<840/μl, n = 79) ($p = 0.26$, Fig. 1F). We also observed that serum CRP concentration was higher in patients with NLR ≥4 than with NLR <4 (Table 2). This finding suggested that the association between NLR and HCC recurrence was due to inflammatory cytokines rather than depletion of lymphocytes.

Although CRP concentration was associated with NLR, CRP concentration itself did not statistically affect RFS (Supplementary Fig. 2A and B). When we divided patients into two groups relative to various cut-offs for neutrophil and lymphocyte counts, we found that a neutrophil count ≥2000/μl was negatively correlated with HCC recurrence after LDLT (Supplementary Fig. 2C), whereas a neutrophil count ≥3000/μl was not correlated with RFS (Supplementary Fig. 2D). Lymphocyte counts of <600/μl (Supplementary Fig. 2E) and <500/μl (Supplementary Fig. 2F) did not correlate with RFS.

VEGF and IL-8 expression did not correlate with NLR

To determine whether elevated neutrophil levels were a primary source of VEGF and IL-8, the major angiogenesis or tumor growth factors, we measured the intra and peritumoral levels of *VEGF* and *IL-8* mRNAs. We found that neither intra nor peritumoral expression of *VEGF* and *IL-8* mRNA was correlated with NLR (Supplementary Fig. 4A and B). Moreover, ELISA assays of serum VEGF and IL-8 showed that they did not correlate with NLR (Supplementary Fig. 4C) either. Taken together, these findings indicate that none of these angiogenesis and tumor growth factors were involved in the mechanism by which NLR correlated with HCC recurrence after LDLT.

IL-17 expression significantly correlated with NLR

To determine whether IL-17 is involved in the relationship between NLR and HCC recurrence, we measured the expression of this cytokine. Since IL-17 is produced by some helper T cells (Th17 cells), not by hepatocytes, little *IL-17* mRNA is present in RNA extracted from liver tissue (data not shown). We therefore used immunohistochemical staining to investigate the intra and peritumoral expression of IL-17. Most IL-17-producing cells were present in the peritumoral region (Fig. 2A). Although intratumoral IL-17 expression did not differ between the high- and low-NLR groups ($p = 0.32$), peritumoral IL17 expression was significantly higher in the high-NLR group ($p = 0.03$, Fig. 2B). Furthermore,

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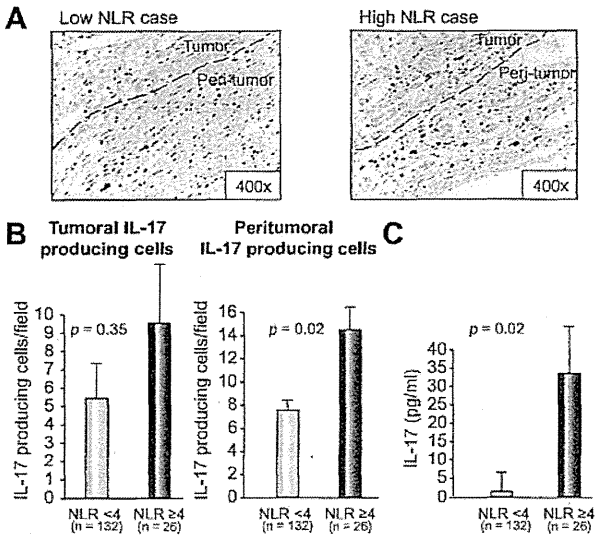

Fig. 2. Hepatic and systemic IL-17 production relative to NLR. (A) Immunohistochemical staining for IL-17-producing cells in paraffin-embedded blocks of liver tissue samples. The left panel shows a sample with low NLR and the right panel shows a sample with high NLR. In both groups, there were more IL-17-producing cells in peritumoral than intratumoral regions. (B) Count of IL-17-producing cells according to NLR in intratumoral and peritumoral regions. (C) IL-17 concentration in sera collected at the time of transplantation from patients with high and low NLR. (This figure appears in color on the web.)

serum IL-17 concentration was significantly higher in the high- than in the low-NLR group (1.3 *vs.* 33.6 pg/ml, $p = 0.02$, Fig. 2C). These findings indicated that the proinflammatory cytokine IL-17 was significantly associated with NLR.

Next, the expression of IL-17 was compared between patients who had received pre-transplant treatment for HCC and those who had not. The tumoral, peritumoral, or serum IL-17 expression was not different between the two groups (data not shown).

Tumor-associated activation of macrophages is upregulated in the high-NLR group

We also investigated the correlation of NLR with CD163-positive tumor associated macrophages (TAMs). The density of CD68, a marker ubiquitously expressed on macrophages, was not associated with NLR, either in or around the tumors (Fig. 3A). However, the number of CD163-positive TAMs around, but not within, the tumor was significantly higher in the high-NLR group (Fig. 3B). Moreover, the density of TAMs correlated significantly with that of IL-17-producing cells ($R^2 = 0.17$, $p = 0.04$, Fig. 3C). The expression of TAMs was not associated with whether patients had received pretransplant therapies or not (data not shown).

TAMs have recently been found to originate from splenic monocytes [17]. Although the RFS outcomes were similar in recipients who had ($n = 94$) and had not ($n = 64$) undergone splenectomy, the 1-, 3-, and 5-year RFS rates in the 19 patients with high NLR who had undergone splenectomy (88.5%, 68.1%, and 33.3%, respectively) were significantly higher than in the 7 patients with high NLR who had not undergone splenectomy (68.1%, 50.3%, and 16.7%, respectively; $p = 0.02$, Fig. 4). In the low-NLR group, there was no difference in HCC recurrence between the 75 patients who had undergone splenectomy and the 57 who had not ($p = 0.63$, Supplementary Fig. 4).

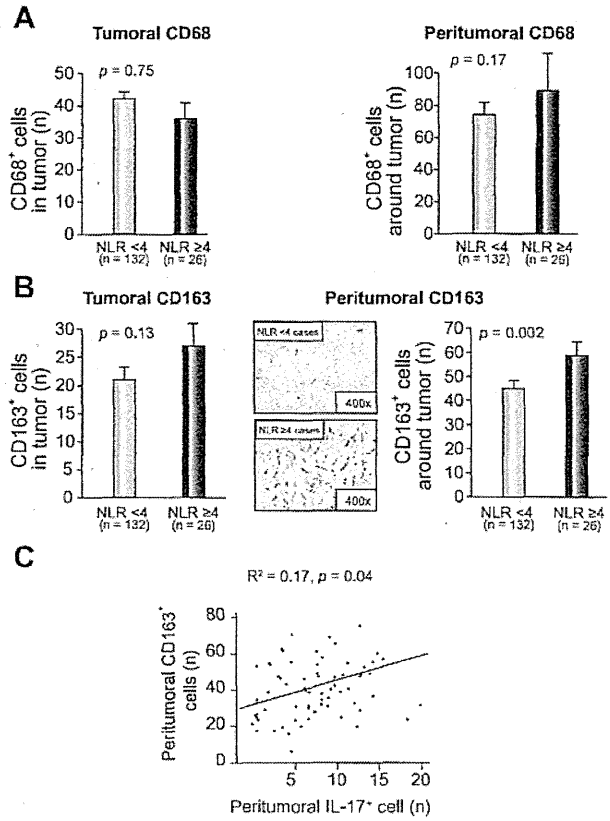

Fig. 3. Immunohistochemical assays for CD68- and CD163-positive macrophages. (A) Cells positive for CD68, a ubiquitously expressed macrophage marker, were counted in the tumor and peritumoral region. (B) Cells positive for CD163 (TAM marker) were counted in the tumor. CD163 immunohistochemical staining of the peritumoral region of samples with low and high NLR. Staining was greater in the high-NLR group ($p = 0.005$.). (C) Relationship between the density of CD163-positive cells and IL-17-producing cells in the peritumoral region ($R^2 = 0.17$, $p = 0.04$). (This figure appears in color on the web.)

Discussion

Many studies to date have shown that higher NLR is correlated with adverse survival outcomes in patients with various solid tumors [8–12,18]. Despite the total replacement of the liver, HCC recurrence following DDLT was correlated with pretransplant NLR [12,18]. To expand these findings, we assessed whether pretransplant NLR was correlated with HCC recurrence after LDLT. We found that NLR ⩾4 showed the greatest correlation with recurrence; in contrast, other studies have used NLR ⩾5 as the cut-off value [8–12,18]. Moreover, to our knowledge, this study is the first to describe the molecular mechanism involved in the relationship between NLR and cancer recurrence.

Previous studies [8–12] have shown that high NLR reflects relatively depleted lymphocytes, impairing the host immune response to malignancy. Elevated neutrophils were regarded as a reservoir of VEGF. In contrast, we found that the lymphocyte number was not associated with survival outcomes, whereas the neutrophil count was. Furthermore, the expression of tumoral, peritumoral, and circulating VEGF did not show any correlation with NLR. We also found that expression of IL-8,

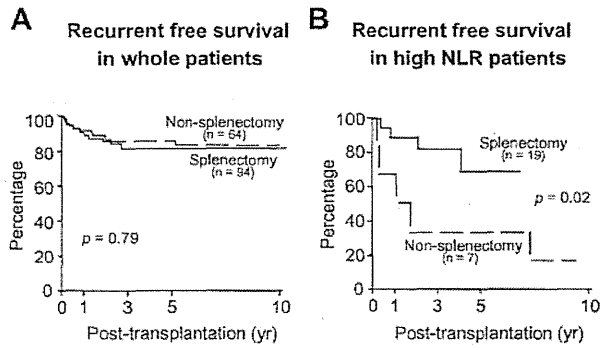

Fig. 4. Relationships between RFS and splenectomy. (A) RFS rates in patients who had and had not undergone splenectomy. (B) RFS rates in patients with high NLR who had and had not undergone splenectomy.

another angiogenesis and tumor growth factor that can promote neutrophil recruitment [19], was not associated with HCC recurrence after LDLT. In contrast, we found that serum CRP concentration was positively correlated with NLR. Taken together, these results indicate that elevated NLR promotes HCC recurrence via some sort of inflammatory microenvironment, not via angiogenesis alone.

IL-17 is a proinflammatory cytokine that promotes HCC growth [20,21]. In addition, IL-17 is an initiator of neutrophil recruitment by CXC chemokines, such as CCL2 released from IL-17-producing T cells [15,21,22]. We observed a correlation between elevated NLR and upregulation of IL-17 production in both peritumoral regions of the liver and peripheral blood. IL-17 may therefore be a key molecule involved in the relationship between NLR and HCC recurrence.

TAMs have been reported to be a major component of the tumor inflammatory microenvironment and to promote proliferation and tumor angiogenesis [16]. Monocytes are recruited from the circulation into local tissue or malignant sites, where they are recognized by CD68-expressing residential macrophages. In response to inflammatory cytokines released by tumors, some of these residential macrophages differentiate into CD163-expressing TAMs. In contrast to CD68-positive macrophages, CD163-positive TAMs are suppressors of the antitumor immune response. Furthermore, IL-17-producing cells have been found to interact with TAMs in HCC patients [20,23]. We observed a correlation between IL-17-producing cells and the density of CD163, confirming their collaboration. Interestingly, both IL-17 producing cells and CD163 positive TAMs produce the same family of CXC chemokines that promote the recruitment of monocytes and neutrophils [21,24,25]. Moreover, both cell types promote tumor migration mediated by matrix metalloproteinase [26,27] and downregulate the antitumor immune response resulting from the expansion of FoxP3-positive regulatory T cells [25,26] or programmed death-1-positive T cells [28,29].

In summary, IL-17-producing T cells are thought to release CXC chemokines that recruit neutrophils, leading to elevated NLR, and promote the differentiation of tissue macrophages in peritumoral regions into TAMs. Both IL-17-producing cells and TAMs accelerate tumor progression and antitumor T cell exhaustion. Our findings and other studies [12,18] demonstrate the association between elevated NLR and HCC recurrence in LT recipients, from whom tissue macrophages and IL-17-producing T cells in the liver have been completely removed. However, elevated preoperative serum IL-17 has also been found to promote tumor recurrence [30]. Circulating IL-17 may recruit TAMs into sites of tumor recurrence even after LT. Recurrent HCC following LT may be an indication for resection, but it remains unclear whether TAMs are involved at recurrent sites, suggesting the need for additional investigation using animal models. Monocytes that differentiate into TAMs have been recently reported to originate from the spleen [17]. We found that RFS rates were significantly lower in LT recipients with high NLR who had not undergone splenectomy than those who had, suggesting the continuous feeding of splenic TAMs with high IL-17 concentrations following LT. Although investigations involving larger numbers of patients are required, our findings suggest that splenectomy may be a useful strategy for preventing tumor recurrence after LT in HCC patients with high NLR.

In conclusion, we found that elevated NLR was significantly correlated with HCC recurrence after LDLT via an inflammatory tumor microenvironment provided by TAMs and IL-17-producing cells.

Conflict of interest

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

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.08.017.

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REVIEW

Role of miR-122 and lipid metabolism in HCV infection

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Abstract Hepatitis C virus (HCV) exhibits a narrow host range and a specific tissue tropism. Mice expressing major entry receptors for HCV permit viral entry, and therefore the species tropism of HCV infection is considered to be reliant on the expression of the entry receptors. However, HCV receptor candidates are expressed and replication of HCV-RNA can be detected in several nonhepatic cell lines, suggesting that nonhepatic cells are also susceptible to HCV infection. Recently it was shown that the exogenous expression of a liver-specific microRNA, miR-122, facilitated the efficient replication of HCV not only in hepatic cell lines, including Hep3B and HepG2 cells, but also in nonhepatic cell lines, including Hec1B and HEK-293T cells, suggesting that miR-122 is required for the efficient replication of HCV in cultured cells. However, no infectious particle was detected in the nonhepatic cell lines, in spite of the efficient replication of HCV-RNA. In the nonhepatic cells, only small numbers of lipid droplets and low levels of very-low-density lipoprotein-associated proteins were observed compared with findings in the hepatic cell lines, suggesting that functional lipid metabolism participates in the assembly of HCV. Taken together, these findings indicate that miR-122 and functional lipid metabolism are involved in the tissue tropism of HCV infection. In this review, we would like to focus on the role of miR-122 and lipid metabolism in the cell tropism of HCV.

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Introduction

More than 170 million individuals worldwide are chronically infected with hepatitis C virus (HCV), and the cirrhosis and hepatocellular carcinoma (HCC) induced by HCV infection are life-threatening diseases [1]. On the other hand, HCV infection sometimes induces extra-hepatic manifestations (EHM), including mixed cryoglobulinemia and non-Hodgkin lymphoma [2–5]. The mechanisms of the pathogenesis and cell tropism of HCV have not been fully elucidated yet owing to the lack of an appropriate infection model. Although chimpanzees are susceptible to HCV infection, the use of these animals to study experimental infection is ethically problematic, and no other animal model with susceptibility to HCV infection has been established [6]. Furthermore, robust in vitro HCV propagation has been limited to the combination of cell-culture-adapted clones based on the genotype 2a JFH1 strain (HCVcc) and human liver cancer-derived Huh7 cells [7, 8]. The expression of a liver-specific microRNA, miR-122, has been shown to dramatically enhance the translation and replication of HCV-RNA [9]. Recently, several reports have shown that the exogenous expression of miR-122 facilitates the efficient replication of viral RNA in several hepatic and nonhepatic cell lines [10–13]. Of note, the clinical application of a specific inhibitor of miR-122 to chronic hepatitis C patients is now in progress [14]. In addition, it has been shown that liver-specific expression of very-low-density lipoprotein (VLDL)-associated proteins is involved in the assembly of infectious HCV particles [15, 16]. This review will focus on the role of miR-122 expression and lipid metabolism in HCV infection.

microRNA and virus infection

miRNAs were first identified by Lee et al. [17] and since that time a great number of miRNAs have been registered in the miRNA database. miRNA incorporated into RNA-induced silencing complex (RISC) interacts with a target mRNA via a specific recognition element. RISC contains argonaute 2 (Ago2), Dicer, and TAR RNA binding protein (TRBP) [18, 19]. In humans, Ago2 plays a pivotal role in the repression of translation of target genes [20]. It is now commonly believed that miRNAs play important roles in cell homeostasis, and that abnormality of miRNA expression participates in the development of several diseases, including viral infections [18, 19]. miRNAs encoded by Epstein–Barr virus (EBV) were identified in 2004 [4, 21], and over 200 viral miRNAs have been reported in several DNA viruses, especially in herpesviruses [22, 23]. Previous reports have shown that viral miRNAs participate in viral propagation by regulating the host gene expression [22, 23]. Many viral miRNAs suppress the host gene expression involved in innate and acquired immunities and enhance viral propagation [22, 24, 25]. Most RNA viruses replicate in the cytoplasm, and thus it had been believed that RNA viruses do not encode viral miRNAs. Rouha et al. [26] showed that an RNA virus, the tick-borne encephalitis virus, is capable of producing functional miRNA by the insertion of an miRNA element into viral RNA. Actually, it has been shown that virus-derived small RNAs emerge by infection with RNA viruses, including influenza virus and West Nile virus [27, 28]. These data suggest that both viral-encoded and host gene-derived miRNAs are involved in the regulation of viral propagation.

Liver-specific microRNA, miR-122

miR-122 is a liver-specific microRNA and is the microRNA most abundantly expressed in the liver [29–31]. Although Li et al. [32] have suggested that hepatocyte nuclear factor 4 alpha (HNF4A) positively regulates the expression of miR-122, the details on the tissue specificity of miR-122 expression have not been fully elucidated yet. miR-122 targets the 3′untranslated region (3′UTR) of the mRNAs of cytoplasmic polyadenylation element binding protein (CPEB), hemochromatosis (Hfe), hemojuverin (Hjv), disintegrin, and metalloprotease family 10 (ADAM10) and represses their translation [33–35]. miR-122 activates the translation of p53 mRNA through the suppression of CPEB and participates in cellular senescence [33]. Through the inhibition of Hfe and Hjv, miR-122 participates in iron metabolism [34]. Esau et al. [36] showed that miR-122 positively regulated lipid metabolism through the reduction of the mRNAs of lipid-associated proteins, and that inhibition of miR-122 expression attenuated liver steatosis in high-fat-fed mice, suggesting that miR-122 may be an attractive therapeutic target for metabolic diseases. miR-122 has also been shown to be involved in the propagation of hepatitis viruses, including hepatitis B virus (HBV) and HCV [9, 37, 38]. Wang et al. [38] have revealed that miR-122 suppresses cyclin G1, and this factor is known to enhance the replication of HBV by inhibiting the binding of p53 to HBV enhancer elements. In other reports, a low level of miR-122 expression in plasma was significantly associated with the incidence of HBV-related HCC [39]. These results suggest that miR-122 expression inhibits the propagation and pathogenesis of HBV. On the other hand, miR-122 expression enhances the propagation of HCV through genetic interaction with the 5′UTR of the HCV genome [9]. It is interesting to note that the effects of miR-122 expression on viral propagation are different between HBV and HCV.

miR-122 expression and HCV infection (Fig. 1)

Jopling et al. [9] reported for the first time that the inhibition of miR-122 dramatically decreased RNA replication in HCV replicon cells harboring subgenomic (SGR) or fullgenomic (FGR) viral RNA. They identified the 21 nucleotide (nt) of the miR-122 binding site in the 5′ end of the 5′UTR of HCV RNA. In addition, lack of enhancement of HCV replication by the expression of a mutant miR-122 incapable of binding to the 5′UTR was canceled by the introduction of a complementary mutation in the 5′UTR, suggesting that direct interaction of miR-122 with the 5′UTR is crucial for the enhancement of HCV replication. In subsequent reports, they identified a second adjacent miR-122 binding site in the 5′UTR [40]. Furthermore, ectopic expression of the mutant miR-122 rescued the replication of an HCV RNA possessing mutations in both miR-122 binding sites, suggesting that the interaction of miR-122 with both sites in the 5′UTR is required to augment viral replication. In addition, Machlin et al. [41] have revealed that not only the seed sequence but also nucleotides located at the positions of 15 and 16 in miR-122 are required for the enhancement of HCV replication. Interestingly, nucleotides 15 and 16 are not required for the conventional microRNA function of miR-122, suggesting that the conventional machinery of miR-122 is not involved in the miR-122-dependent enhancement of HCV replication. A recent study showed that the interaction of miR-122 with the 5′UTR of HCV was also required for the efficient production of infectious particles in cell culture [42].

Although the precise mechanisms of the miR-122-mediated enhancement of HCV replication have not been

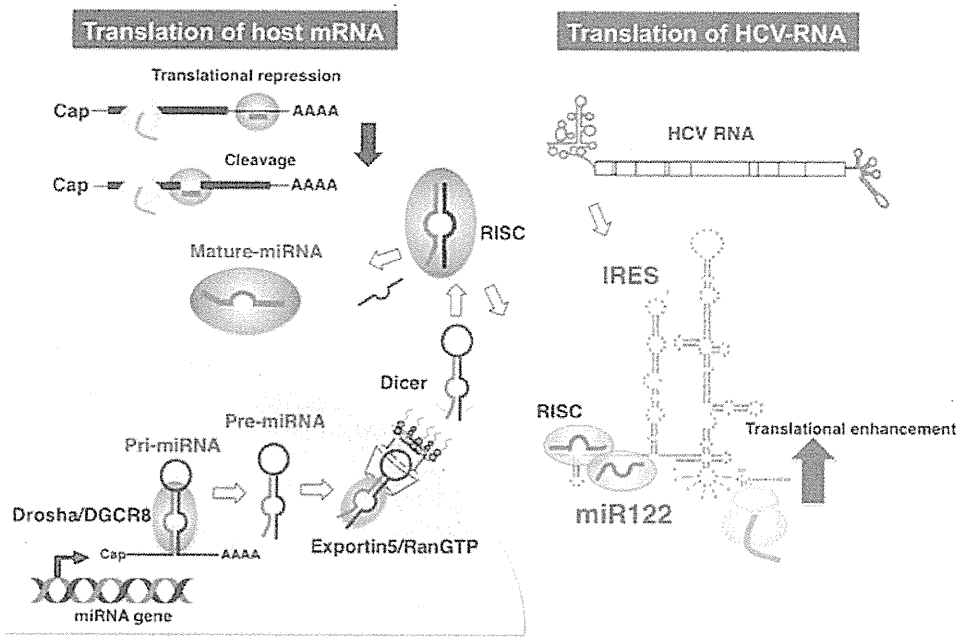

Fig. 1 miR-122 enhances the translation of hepatitis C virus (*HCV*) RNA. Primary miRNA (*pri-miRNA*) transcribed by RNA polymerase II in the nucleus is processed into precursor miRNA (*pre-miRNA*) by Drosha and DiGeorge syndrome critical region protein 8 (*DGCR8*). Pre-miRNA is exported into the cytoplasm by nucleocytoplasmic shuttle protein exportin 5, processed to 22nt by dicer, and then incorporated into argonaute proteins to form the RNA-induced silencing complex (*RISC*). The passenger strand of miRNA (*blue*) is degraded and the guide strand (*red*) is matured in the RISC. Generally, miRNA represses the translation of host mRNA by binding to its 3'untranslated region (3'UTR). In contrast, liver-specific miR-122 binds to two sites in the 5'UTR of the HCV genome and enhances its translation and replication. *GTP* Guanosine-5-triphosphate, *IRES* internal ribosomal entry site

fully elucidated yet, Henke et al. [43], by using polymerase defective viral RNA, showed that miR-122 stimulated the translation of HCV RNA by enhancing the association of ribosomes at an early initiation stage. They concluded that miR-122 might contribute to HCV liver tropism at the level of translation. Wilson et al. [44] showed that knockdown of Ago2 in SGR cells and HCVcc-infected cells attenuated HCV replication, and that knockdown of Ago2 also reduced the translation of the polymerase defective HCV RNA. Shimakami et al. [45] showed that miR-122 stabilized viral RNA and reduced its decay in concert with Ago2, and that miR-122-dependent stabilization of HCV RNA was not observed in Ago2-knockout murine embryonic fibroblasts. These results suggest that Ago2 is required for the efficient enhancement of both the translation and replication of HCV. On the other hand, Machlin et al. [41] have suggested that the 3′ overhang binding of miR-122 to the 5′ end of the HCV genome participates in circumvention from the recognition by the cytoplasmic RNA sensor, RIG-I. It is feasible to speculate that miR-122 has other functions in the HCV life cycle, in addition to the stabilization of viral RNA and evasion from the host's innate immune response.

Establishment of new permissive cell lines for HCV propagation by the expression of miR-122

The lack of immunocompetent small animal models and cell culture systems to support the propagation of HCV in patient sera has hampered both the understanding of the HCV life cycle and the development of antiviral drugs [46]. HCV replicon cells in which the HCV genome autonomously replicates, and pseudotype viruses bearing HCV E1 and E2 glycoproteins were established to assess viral replication and entry, respectively [47, 48]. Afterwards, an infectious HCV derived from the JFH1 strain of genotype 2a (HCVcc) was developed [7, 8]. On the basis of the data obtained from these in vitro systems, the HCV life cycle has been clarified, and host factors involved in HCV propagation have been identified as therapeutic targets for chronic hepatitis C [46]. However, the robust propagation of HCVcc in well-characterized human liver cell lines other than Huh7 had not been successful until recently. Chang et al. [49] showed that the exogenous expression of miR-122 facilitated the replication of HCV RNA in kidney-derived HEK-293 cells. In addition, Lin et al. have demonstrated that the expression of miR-122 and depletion of interferon regulatory factor 3 (IRF-3) permit replication

of the HCV genome in mouse fibroblasts [50]. These results suggest that the expression of miR-122 might facilitate the efficient replication of HCVcc not only in hepatic cells but also in nonhepatic cells. In fact, the expression level of miR-122 in Huh7 cells has been shown to be higher than that in other hepatic cell lines, including Huh6, HepG2, and Hep3B cells [10]. Recently, two groups reported that miR-122 expression facilitated the efficient propagation of HCVcc in human hepatic cell lines [10, 11]. Narbus et al. [11] showed that HepG2 cells stably expressing CD81 and miR-122 supported efficient replication and the production of infectious particles. Interestingly, internal ribosomal entry site (IRES)-dependent translation of HCV exhibited a slight (1.4–2.1-fold) increase by the expression of miR-122 in HepG2 cells compared with that in parental cells, suggesting that miR-122 is required for efficient RNA replication but not in translation in HepG2 cells upon infection with HCVcc. Kambara et al. [10] established a novel permissive cell line for the propagation of HCVcc by the expression of miR-122 in Hep3B cells. miR-122 expression facilitated the efficient propagation of HCVcc and the establishment of HCV replicon cells in Hep3B cells. In addition, "cured" Hep3B cells established by the elimination of HCV RNA from the Hep3B replicon cells facilitated the efficient propagation of HCVcc compared to parental cells. Interestingly, the expression of miR-122 in the "cured" Hep3B cells was significantly higher than that in the parental cells. In addition, Ehrhadt et al. [51] have shown that the expression levels of miR-122 in Huh7-derived cured cells, including Huh7.5 and Huh-Lunet cells, are significantly higher than those in parental Huh7 cells. Collectively, these results suggest that miR-122 is a key determinant of the efficient replication of HCVcc in hepatic cell lines.

Expression of miR-122 facilitates the efficient replication of HCV in nonhepatic cells

In clinical studies, negative strands of HCV genome have been detected in nonhepatic tissues of chronic hepatitis C patients, suggesting the possibility of extrahepatic propagation of HCV [52–56]. In addition, HCV replication was detected in peripheral blood mononuclear cells (PBMCs) of patients with occult HCV infection [57]. Roque-Afonso et al. [52] showed that highly divergent variants of HCV were detectable in PBMCs, but not in plasma or in liver, suggesting the possibility of the extrahepatic propagation of HCV. Furthermore, previous reports have suggested that recurrences of HCV infection after antiviral treatment or liver transplantation were attributable to chronic infection of HCV in extrahepatic tissues [58]. Collectively, these results might suggest a correlation between extrahepatic HCV replication and the development of EHM, including mixed cryoglobulinemia and non-Hodgkin lymphoma, which are frequently observed in chronic hepatitis C patients. However, details of the extrahepatic propagation of HCV have not been studied owing to the lack of an appropriate experimental model [59, 60].

HCV replicon cells have been established in several nonhepatic cell lines. Kato et al. [61] established JFH1-based SGR cells by using HeLa and HEK293 cells, suggesting that the HCV genome can replicate in nonhepatic cells. In addition, Fletcher et al. [62] showed that brain endothelial cells supported HCV entry and replication, suggesting that HCV infection in the central nervous system participates in HCV-associated neuropathologies. Given the marked effects of miR-122 expression on the propagation of HCVcc in hepatic cell lines, we hypothesized that the expression of miR-122 in nonhepatic cell lines would facilitate the establishment of novel permissive cell lines for HCV. Recently, we have shown that Hec1B cells derived from the human uterus exhibited a low level of viral replication and the exogenous expression of miR-122 significantly enhanced replication upon infection with HCVcc [63]. In addition, an miR-122-specific inhibitor for miR-122 called locked nucleic acid (LNA-miR-122) inhibited the enhancement of HCVcc replication in Hec1B cells expressing miR-122, while the basal replication of HCVcc in parental Hec1B cells was resistant to the treatment. These results suggest that Hec1B cells permit HCV replication in an miR-122-independent manner and the exogenous expression of miR-122 enhances viral replication. In this report, cured Hec1B cells established by the elimination of HCV RNA from Hec1B replicon cells exhibited more potent replication of HCVcc than the parental cells. As seen in the cured Hep3B cells, the expression levels of miR-122 in the Hec1B cured cells were significantly higher than those in the parental cells [63]. Taken together, these results show that the expression of miR-122 facilitates the replication of HCVcc in nonhepatic cells.

Viral assembly in nonhepatic cells

Previous reports have shown that the production of VLDL is involved in the formation of infectious HCV particles [15, 16]. Apolipoprotein B (ApoB), apolipoprotein E (ApoE), and microsomal triglyceride transfer protein (MTTP) have major roles in the secretion of VLDL. Gastaminza et al. [15] have demonstrated that ApoB and MTTP are cellular factors essential for the efficient assembly of infectious HCV particles. They concluded that HCV acquired hepatocyte tropism through utilization of the VLDL secretory pathway. On the other hand, studies by

other groups have demonstrated that infectious HCV particles are highly enriched in ApoE, which is a major determinant of HCV infectivity and production [64]. In their reports, small interfering RNA (siRNA)-mediated knockdown of ApoB and treatment with MTTP inhibitors exhibited no significant effect on the infectivity and production of HCV, suggesting that ApoE but not ApoB is required for viral assembly. In addition, Mancone et al. [65] have shown that apolipoprotein A-I (ApoA-I) is required for the replication of HCV and the production of infectious particles. Collectively, these results suggest that several VLDL-associated proteins are involved in HCV assembly.

In our recent report, the viral assembly process was shown to be impaired in nonhepatic cells exogenously expressing miR-122, in spite of the efficient replication of the HCV genome [63]. Interestingly, low but substantial infectious titers were detected in hepatic Hep3B cells upon infection with HCVcc, even though the RNA replication was lower than that in nonhepatic Hec1B cells expressing miR-122. The expression levels of VLDL-associated proteins, including ApoE, ApoB, and MTTP, in nonhepatic cell lines were significantly lower than those in hepatic cell lines, suggesting that lack of expression of VLDL-associated proteins is one of the reasons for the inability of nonhepatic cells to produce infectious particles. Miyanari et al. [66] showed that lipid droplets (LDs) were required for the formation of infectious particles via interaction between the core protein and viral RNA. Interestingly, only a small amount of LDs was detected in nonhepatic cells, including Hec1B and HEK293T cells, compared with the amount in hepatic cell lines, suggesting that a low level of LD formation is also involved in the impairment of infectious particle formation in nonhepatic cells [63]. Taken together, these findings suggest the possibility that the reconstitution of functional lipid metabolism in nonhepatic cells facilitates the production of infectious particles.

Tropism of HCV infection

In many cases, the cell tropism of viral infection is defined by the expression of virus-specific receptors. The expression of CD4 and chemokine receptors has an important role in the determination of the lymphotropism of human immunodeficiency virus infection [67]. In measles virus infection, the signaling lymphocyte activation molecule is a determinant of lymphotropism [68, 69]. Previous reports have shown that human CD81, scavenger receptor class B1 (SR-B1), Claudin1 (CLDN1), and Occludin (OCLN) are crucial for HCV entry [70–73]. Although murine cells cannot permit HCV entry, the exogenous expression of human-derived receptor candidates in murine cells has been shown to facilitate HCV entry, suggesting that HCV-specific receptors participate in the determination of the cell tropism of HCV [74, 75]. However, previous reports have also revealed that HCV receptor candidates were highly expressed in many nonhepatic tissues [62, 76], and our recent report has demonstrated that many nonhepatic cells permit the entry of HCV pseudotypes [63]. In addition, many reports have suggested the possibility of HCV replication in extrahepatic sites such as PBMCs and neuronal cells [55, 62], suggesting that host factors other than receptors could be involved in the tissue tropism of HCV.

Although previous reports have shown that host factors such as VAMP-associated protein (VAP)-A, VAP-B, cyclophilin A, FK506 binding protein 8, and heat shock protein 90 participate in HCV replication, these molecules are unlikely to participate in the determination of the liver tropism of HCV, owing to their ubiquitous expression [46, 77–79]. As described above, miR-122 is abundantly expressed specifically in hepatocytes and is essential for the efficient replication of HCV. In addition, a recent report showed that hepatocyte-like cells derived from induced pluripotent stem cells (iPSCs) expressed high levels of miR-122 and supported the entire life cycle of HCVcc, suggesting that miR-122 might be one of the most critical determinants of the liver tropism of HCV infection [80, 81]. On the other hand, VLDL-associated proteins,

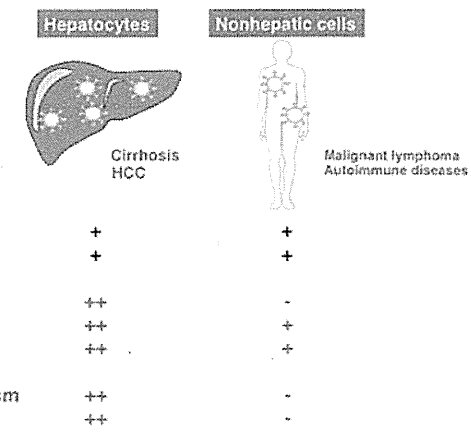

Fig. 2 HCV replication in hepatocytes and nonhepatic cells. Chronic HCV infection induces liver cirrhosis and hepatocellular carcinoma (*HCC*), and is also often associated with the development of extrahepatic manifestations (EHM) such as malignant lymphoma and autoimmune diseases. Not only hepatocytes but also nonhepatic cells express major HCV receptors, including CD81, SR-BI, CLDN1, and OCLN. In hepatocytes, functional expression of miR-122 and lipid metabolism facilitate the efficient propagation of HCV. In contrast, the lack of expression of miR-122 and very-low-density lipoprotein (VLDL)-associated proteins might be associated with the incomplete propagation of HCV in nonhepatic cells. Low levels of HCV replication in nonhepatic cells may participate in the development of EHM

including ApoB, ApoE, and MTTP, are specifically expressed in hepatic cells, and no infectious particles are produced in nonhepatic cells such as Hec1B and 293T-CLDN cells [63]. Collectively, these data suggest that the VLDL-producing system is involved in the liver tropism of HCV.

Although HCV can internalize not only into hepatocytes but also into nonhepatic cells through receptor-mediated endocytosis, miR-122 expression and functional lipid metabolism in hepatocytes facilitate the efficient replication and assembly of HCV (Fig. 2). On the other hand, lack of expression of miR-122 and VLDL-associated proteins might be associated with the incomplete propagation of HCV in nonhepatic cells (Fig. 2).

Conclusion

Recent progress in HCV research has revealed that the tissue tropism of HCV is reliant on the expression of liver-specific miR-122 and a functional lipid metabolism rather than being reliant on the expression of entry receptors. However, the molecular mechanisms of the enhancement of viral replication induced by the interaction of miR-122 with the 5′UTR of HCV and the assembly of viral particles via VLDL-producing machinery remain unknown. In addition, the participation of nonhepatic cells in the development of EHM has been suggested, through an incomplete or low level of HCV replication. Elucidation of the liver tropism of HCV will provide a clue to the development of new antiviral drugs for the treatment of chronic hepatitis C and could lead to an understanding of the pathogenesis of EHM induced by HCV infection.

Conflict of interest The authors declare that they have no conflicts of interest.

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Expression of MicroRNA miR-122 Facilitates an Efficient Replication in Nonhepatic Cells upon Infection with Hepatitis C Virus

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Hepatitis C virus (HCV) is one of the most common etiologic agents of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma. In addition, HCV infection is often associated with extrahepatic manifestations (EHM), including mixed cryoglobulinemia and non-Hodgkin's lymphoma. However, the mechanisms of cell tropism of HCV and HCV-induced EHM remain elusive, because *in vitro* propagation of HCV has been limited in the combination of cell culture-adapted HCV (HCVcc) and several hepatic cell lines. Recently, a liver-specific microRNA called miR-122 was shown to facilitate the efficient propagation of HCVcc in several hepatic cell lines. In this study, we evaluated the importance of miR-122 on the replication of HCV in nonhepatic cells. Among the nonhepatic cell lines expressing functional HCV entry receptors, Hec1B cells derived from human uterus exhibited a low level of replication of the HCV genome upon infection with HCVcc. Exogenous expression of miR-122 in several cells facilitates efficient viral replication but not production of infectious particles, probably due to the lack of hepatocytic lipid metabolism. Furthermore, expression of mutant miR-122 carrying a substitution in a seed domain was required for efficient replication of mutant HCVcc carrying complementary substitutions in miR-122-binding sites, suggesting that specific interaction between miR-122 and HCV RNA is essential for the enhancement of viral replication. In conclusion, although miR-122 facilitates efficient viral replication in nonhepatic cells, factors other than miR-122, which are most likely specific to hepatocytes, are required for HCV assembly.

More than 170 million individuals worldwide are infected with hepatitis C virus (HCV), and cirrhosis and hepatocellular carcinoma induced by HCV infection are life-threatening diseases (57). Although therapy combining pegylated interferon (IFN) and ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotype 1 (37), a more effective therapeutic modality for HCV infection is needed (46). The establishment of *in vivo* and *in vitro* infection systems has been hampered by the narrow host range and tissue tropism of HCV. Although the chimpanzee is the only experimental animal susceptible to HCV infection, it is difficult to use the chimpanzee in experiments due to ethical concerns (3). Furthermore, robust *in vitro* HCV propagation is limited to the combination of cell culture-adapted clones based on the genotype 2a JFH1 strain (HCVcc) and human hepatoma cell lines, including Huh7, Hep3B, and HepG2 (29, 43, 62).

It is well-known that HCV mainly infects hepatocytes. However, the precise mechanism underlying the liver tropism of HCV has not been clarified. Chronic hepatitis C virus infection is often associated with at least one extrahepatic manifestation (EHM), including mixed cryoglobulinemia, non-Hodgkin's lymphoma, lichen planus, thyroiditis, diabetes mellitus, Sjögren syndrome, and arthritis (19). EHMs are frequently more serious than hepatic disease in some patients and sometimes occur even in patients with persistently normal liver functions (19). Mixed cryoglobulinemia is the most-well-characterized HCV-associated disease and is curable by viral clearance through antiviral therapies (6). Although replication of HCV RNA in peripheral blood mononuclear cells (PBMCs) and neuronal cells at a low level was suggested (64), the biological significance of the extrahepatic replication of HCV, particularly in the development of EHMs, is not well understood.

MicroRNAs (miRNAs) are small noncoding RNAs consisting of 20 to 25 nucleotides that modulate gene expression in plants and animals (1, 24). Most miRNAs negatively regulate translation through the interaction with the 3′ untranslated region (UTR) of mRNA in a sequence-specific manner. miRNA 122 (miR-122) is liver specific, is the most abundantly expressed miRNA in the liver, and represses the translation of several mRNAs (5, 7). Jopling et al. reported for the first time that the inhibition of miR-122 dramatically decreased RNA replication in HCV subgenomic replicon (SGR) cells (28). In addition, several reports revealed that a specific interaction between the seed domain of miR-122 and the complementary sequences in the 5′ UTR of HCV RNA is essential for the enhancement of translation and replication of the HCV genome (21, 25, 27, 36). Endogenous expression levels of miR-122 are significantly higher in Huh7 cells than in other hepatic and nonhepatic cell lines (Fig. 1). In addition, previous reports showed that miR-122 expression enhanced the replication of SGR RNA in human embryonic kidney 293 (HEK293) cells and mouse embryonic fibroblasts (MEFs) (8, 35). Furthermore, it was recently shown that exogenous expression of miR-122 facilitates the efficient propagation of HCVcc in Hep3B and HepG2 cells, which are

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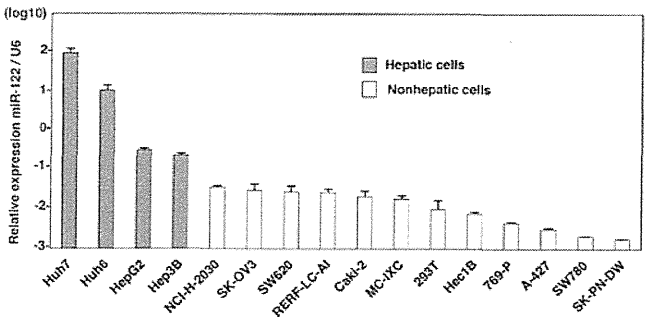

FIG 1 Endogenous expression levels of miR-122 in hepatic and nonhepatic cells. Total miRNAs were extracted from Huh7, Huh6, HepG2, Hep3B, NCI-H-2030, SK-OV3, SW620, RERF-LC-AI, Caki-2, MC-IXC, 293T, Hec1B, 769-P, A-427, SW780, and SK-PN-DW cells, and the expression levels of miR-122 were determined by qRT-PCR.

nonpermissive for HCVcc propagation (29, 43). These results suggest that the high susceptibility of Huh7 cells to the propagation of HCVcc is attributable to the high expression level of miR-122 and raise the possibility of expanding the HCV host range through the exogenous expression of miR-122 in nonhepatic cells.

In this study, we assessed the effect of miR-122 expression on the replication of HCVcc and SGR RNA in several nonhepatic cell lines. Although the exogenous expression of miR-122 in the cell lines facilitates significant RNA replication through a gene-specific interaction between miR-122 and 5′ UTR of HCV RNA, no infectivity was detected in either the cells or the culture supernatants. The current study suggests that the expression of miR-122 plays an important role in HCV cell tropism, as well as in the possible involvement of nonhepatic cells in EHM, through an incomplete propagation of HCV.

MATERIALS AND METHODS

NextBio Body Atlas. The NextBio Body Atlas application presents an aggregated analysis of gene expression across various normal tissues, normal cell types, and cancer cell lines. It enables us to investigate the expression of individual genes as well as gene sets. Samples for Body Atlas data are obtained from publicly available studies that are internally curated, annotated, and processed (31). Body Atlas measurements are generated from all available RNA expression studies that used Affymetrix U133 Plus or U133A Genechip arrays for human studies. The results corresponding to 128 human tissue samples were incorporated from 1,067 arrays, the results corresponding to 157 human cell types were incorporated from 1,474 arrays, and the results corresponding to 359 human cancer cell lines were incorporated from 376 arrays. Gene queries return a list of relevant tissues or cell types rank ordered by absolute gene expression and grouped by body systems or across all body systems. In the current analysis, we screened for nonhepatic cell lines expressing HCV receptor candidates, including CD81, SR-BI, claudin1 (CLDN1), and occludin (OCLN), or very-low-density lipoprotein (VLDL)-associated proteins, including apolipoprotein E (ApoE), ApoB, and microsomal triglyceride transfer protein (MTTP). A detailed analysis protocol developed by NextBio was described previously (31). The raw data used in this application are derived from the GSK Cancer Cell Line data deposited at the National Cancer Institute website (https://array.nci.nih.gov/caarray/project/woost-00041) and additionally from NCBI Gene Expression Omnibus (GEO) accession number GSE5720 for cell lines SK-OV-3 and SW620.

Sample collection and RNA extraction for microarray analysis. Total RNAs extracted from cells were purified by using an miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Eluted RNAs were quantified using a Nanodrop ND-1000 (version 3.5.2) spectrophotometer (Thermo Scientific, Wartham, MA). RNA integrity was evaluated using the RNA 6000 LabChip kit and bioanalyzer (Agilent Technologies, Santa Clara, CA). Each RNA that had an RNA integrity number (RIN) greater than 9.0 was used for the microarray experiments.

Microarray experiment. Expression profiling was generated using the 4 × 44K whole-human-genome oligonucleotide microarray (version 2.0) G4845A (Agilent Technologies). Each microarray uses 44,495 probes to interrogate 27,958 Entrez gene RNAs. One hundred nanograms of total RNA was reverse transcribed into double-stranded cDNAs by AffinityScript multiple-temperature reverse transcriptase and amplified for 2 h at 40°C. The resulting cDNAs were subsequently used for *in vitro* transcription by the T7 polymerase and labeled with cyanine-3-labeled cytosine triphosphate (Perkin Elmer, Waltham, MA) for 2 h at 40°C using a low-input Quick-Amp labeling kit (Agilent Technologies) according to the manufacturer's protocol. After labeling, the rates of dye incorporation and quantification were measured with a Nanodrop ND-1000 (version 3.5.2) spectrophotometer (Thermo Scientific), and then the cRNAs were fragmented for 30 min at 60°C in the dark. Differentially labeled samples of 1,650 ng of cRNA were hybridized on Agilent 4 × 44K whole-genome arrays (version 2.0; 026652; Agilent Design) at 65°C for 17 h with rotation in the dark. Hybridization was performed using a gene expression hybridization kit (Agilent Technologies) following the manufacturer's instructions. After washing in gene expression washing buffer, each slide was scanned with the Agilent microarray scanner G2505C. Feature extraction software (version 10.5.1.1) employing defaults for all parameters was used to convert the images into gene expression data. Raw data were imported into a Subio platform (version 1.12) for database management and quality control. Raw intensity data were normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression levels for further analysis. These raw data have been accepted by GEO (a public repository for microarray data aimed at storing minimum information about microarray experiments [MIAME]).

Plasmids. The cDNA clones of wild-type (WT) and mutant (MT) pri-miR-122 and *Aequorea coerulescens* green fluorescent protein (AcGFP) were inserted between the XhoI and XbaI sites of lentiviral vector pCSII-EF-RfA, which was kindly provided by M. Hijikata, and the resulting plasmids were designated pCSII-EF-WT-miR-122, pCSII-EF-MT-miR-122, and pCSII-EF-AcGFP, respectively. Plasmids pHH-JFH1 and pSGR-JFH1, which encode full-length and subgenomic cDNA of the JFH1 strain, respectively, were kindly provided by T. Wakita. pHH-JFH1-E2p7NS2mt contains three adaptive mutations in pHH-JFH1 (53). pHH-JFH1-M1 and pHH-JFH1-M2 were established by the introduction of a point mutation of nucleotide 26 located in site 1 and nucleotide 41 in site 2 of the 5′ UTR of the JFH1 cDNA construct pHH-JFH1. pSGR-Con1, which encodes SGR of the Con1 strain, was kindly provided by R. Bartenschlager. The complementary sequences of miR-122 were introduced into the multicloning site of the pmirGLO vector (Promega, Madison, WI), and the resulting plasmid was designated pmirGLO-compl-miR-122. The plasmids used in this study were confirmed by sequencing with an ABI 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cell lines. All cell lines were cultured at 37°C under the conditions of a humidified atmosphere and 5% CO_2. The following cells were maintained in Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS): human hepatocellular carcinoma-derived Huh7, Hep3B, and HepG2; embryonic kidney-derived HEK293 and 293T; lung-derived RERF-LC-AI, NCI-H-2030, and A-427; kidney-derived Caki-2 and 769-P; neuron-derived MC-IXC and SK-PN-DW; uterus-derived Hec1B; ovary-derived SK-OV3; colon-derived SW620; and urinary bladder-derived SW780 cells. RERF-LC-AI (RCB0444) cells were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Hec1B (JCRB1193) cells were obtained from the JCRB Cell Bank. NCI-H-2030, A-427, Caki-2, 769-P, MC-IXC, SK-PN-DW, SK-OV3, and SW780 cells were obtained from the American Type Culture Collection (ATCC). SW620 cells were kindly provided by C.

Oneyama. 293T-CLDN cells stably expressing CLDN1 were established by the introduction of the expression plasmids encoding CLDN1 under the control of the CAG promoter of pCAG-pm3. The Huh7-derived cell line Huh7.5.1 was kindly provided by F. Chisari. The Huh7OK1 cell line efficiently propagates HCVcc as previously described (45). Huh7, Hec1B, and HEK293 cells harboring Con1- or JFH1-based HCV SGR were prepared according to the method described in a previous report (47) and maintained in DMEM containing 10% FCS and 1 mg/ml G418 (Nakalai Tesque, Kyoto, Japan).

Antibodies and drugs. Mouse monoclonal antibodies to HCV nonstructural protein 5A (NS5A) and β-actin were purchased from Austral Biologicals (San Ramon, CA) and Sigma-Aldrich, respectively. Mouse anti-ApoE antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-HCV core protein and NS5A were prepared as described previously (41). Rabbit anti-SR-BI antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit anti-CLDN1 and -OCLN antibodies, Alexa Fluor 488 (AF488)-conjugated anti-rabbit or -mouse IgG antibodies, and AF594-conjugated anti-mouse IgG2a antibodies were purchased from Invitrogen (San Diego, CA). Mouse anti-FKBP8 antibody was described previously (44). Mouse anti-double-stranded RNA (anti-dsRNA) IgG2a (J1 and K2) antibodies were obtained from Biocenter Ltd. (Szirak, Hungary). The HCV NS3/4A protease inhibitor was purchased from Acme Bioscience (Salt Lake City, UT). Human recombinant alpha IFN (IFN-α) and cyclosporine were purchased from PBL Biomedical Laboratories (Piscataway, NJ) and Sigma-Aldrich, respectively. BODIPY 558/568 lipid probe was purchased from Invitrogen. The locked nucleic acid (LNA) targeted to miR-122, LNA-miR-122 (5′-CcAttGTcaCaCtCC-3′), and its negative control, LNA-control (5′-CcAttCTgaCcCtAC-3′) (LNAs are in capital letters, DNAs are in lowercase letters; sulfur atoms in oligonucleotide phosphorothioates are substituted for nonbridging oxygen atoms; the capital C indicates LNA methylcytosine), were purchased from Gene Design (Osaka, Japan) (15).

Preparation of viruses. pHH-JFH1-E2p7NS2mt was introduced into Huh7.5.1 cells, HCVcc in the supernatant was collected after serial passages (39), and infectious titers were determined by a focus-forming assay and expressed in focus-forming units (FFUs) (62). Mutant HCVcc was produced from Huh7.5.1 cells expressing MT miR-122 according to the method of a previous report with minor modifications (25). HCVpv, a pseudotype vesicular stomatitis virus (VSV) bearing HCV E1 and E2 glycoproteins, was prepared as previously described (61), and infectivity was assessed by luciferase expression on a Bright-Glo luciferase assay system (Promega), following a protocol provided by the manufacturer and expressed in relative light units (RLUs).

Lipofection and lentiviral gene transduction. Cells were transfected with the plasmids by using Trans IT LT-1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. LNAs were introduced into cells by Lipofectamine RNAiMAX (Invitrogen). The lentiviral vectors and ViraPower lentiviral packaging mix (Invitrogen) were cotransfected into 293T cells, and the supernatants were recovered at 48 h posttransfection. The lentivirus titer was determined by a Lenti-XTM quantitative reverse transcription-PCR (qRT-PCR) titration kit (Clontech, Mountain View, CA), and the expression levels of miR-122 and AcGFP were determined at 48 h postinoculation.

Quantitative RT-PCR. HCV RNA levels were determined by the method described previously (18). Total RNA was extracted from cells by using an RNeasy minikit (Qiagen). The first-strand cDNA synthesis and qRT-PCR were performed with TaqMan EZ RT-PCR core reagents and an ABI Prism 7000 system (Applied Biosystems), respectively, according to the manufacturer's protocols. The primers for TaqMan PCR targeted to the noncoding region of HCV RNA were synthesized as previously reported (42). To determine the expression levels of miR-122, total miRNA was prepared by using the miRNeasy minikit, and miR-122 expression was determined by using fully processed miR-122-specific RT and PCR primers provided in the TaqMan microRNA assays according to the manufacturer's protocol. U6 small nuclear RNA was used as an internal control. Fluorescent signals were analyzed with the ABI Prism 7000 system.

Immunoblotting. Cells were lysed on ice in lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with a protease inhibitor mix (Nacalai Tesque). The samples were boiled in loading buffer and subjected to 5 to 20% gradient SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected with an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Immunofluorescence assay. Cells cultured on glass slides were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at room temperature for 30 min, permeabilized for 20 min at room temperature with PBS containing 0.2% Triton, after being washed three times with PBS, and blocked with PBS containing 2% FCS for 1 h at room temperature. The cells were incubated with PBS containing appropriate primary antibodies at room temperature for 1 h, washed three times with PBS, and incubated with PBS containing AF488- or AF594-conjugated secondary antibodies at room temperature for 1 h. For lipid droplet staining, cells incubated in medium containing 20 μg/ml BODIPY for 20 min at 37°C were washed with prewarmed fresh medium and incubated for 20 min at 37°C. Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). Cells were observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

***In vitro* transcription, RNA transfection, and colony formation.** The plasmids pSGR-Con1 and pSGR-JFH1 were linearized with ScaI and XbaI, respectively, and treated with mung bean exonuclease. The linearized DNA was transcribed *in vitro* by using a MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The *in vitro*-transcribed RNA (10 μg) was electroporated into Hec1B and HEK293 cells at 10^7 cells/0.4 ml under conditions of 190 V and 975 μF using a Gene Pulser apparatus (Bio-Rad, Hercules, CA) and plated on DMEM containing 10% FCS. The medium was replaced with fresh DMEM containing 10% FCS and 1 mg/ml G418 at 24 h posttransfection. The remaining colonies were cloned by using a cloning ring (Asahi Glass, Tokyo, Japan) or fixed with 4% PFA and stained with crystal violet at 4 weeks postelectroporation.

Electron microscopy and correlative FM-EM analysis. Cells were cultured on a Cell Desk polystyrene coverslip (Sumitomo Bakelite) and were fixed with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 7% sucrose. Cells were postfixed for 1 h with 1% osmium tetroxide and 0.5% potassium ferrocyanide in 0.1 M cacodylate buffer (pH 7.4), dehydrated in graded series of ethanol, and embedded in Epon812 (TAAB). Ultrathin (80-nm) sections were stained with saturated uranyl acetate and lead citrate solution. Electron micrographs were obtained with a JEM-1011 transmission electron microscope (JEOL). Correlative fluorescence microscopy (FM)-electron microscopy (EM) allows individual cells to be examined both in an overview with FM and in a detailed subcellular-structure view with EM (51). The NS5A was stained and observed in the Hec1B-derived Con1 SGR cells by the correlative FM-EM method as described previously (44).

Intracellular infectivity. Intracellular viral titers were determined according to a method previously reported (20). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 5 min at 1,000 × *g*. Cell pellets were resuspended in 500 μl of DMEM containing 10% FCS and subjected to three cycles of freezing and thawing using liquid nitrogen and a thermo block set to 37°C. Cell lysates were centrifuged at 10,000 × *g* for 10 min at 4°C to remove cell debris. Cell-associated infectivity was determined by a focus-forming assay.

Statistical analysis. The data for statistical analyses are averages of three independent experiments. Results were expressed as means ± standard deviations. The significance of differences in the means was determined by Student's *t* test.

Microarray data accession number. Access to data concerning this study may be found under GEO experiment accession number GSE32886.

RESULTS

Nonhepatic cell lines susceptible to HCVcc by expression of miR-122. Human CD81 (hCD81), SR-B1, CLDN1, and OCLN are crucial for HCV entry (16, 48, 49, 56). First, we examined the expression of these receptor candidates in nonhepatic cell lines by using the web-based NextBio search engine (Cupertino, CA). Multidimensional scaling was used to visualize the differences in expression patterns of molecules of various tissues, cells, and cell lines from those of hepatic cell lines and primary hepatocytes. We selected nine nonhepatic cell lines as possibly being susceptible to HCVcc infection: NCI-H-2030 (lung), Caki-2 (kidney), 769-P (bladder), A-427 (lung), SK-OV3 (ovary), SW780 (bladder), SW620 (colon), RERF-LC-AI (lung), and Hec1B (uterus) (Fig. 2). In addition, three nonhepatic cell lines previously reported to be susceptible to replication of HCV RNA—that is, SK-PN-DW (neuron), MC-IXC (neuron), and 293T-CLDN (kidney)—were included in this study (8, 17). The expression of each receptor molecule in these 12 nonhepatic cell lines was confirmed by fluorescence-activated cell sorter (FACS) analysis and immunoblotting (Fig. 3A and B). To examine the expression of the functional receptors for HCV entry in these cell lines, we inoculated HCVpv into the cells. Ten of the cell lines (A-427 and SW780 being the exceptions) exhibited various degrees of susceptibility to HCVpv infection (Fig. 3C). Therefore, we examined the possibility of propagation of HCVcc by the expression of miR-122 in these 10 cell lines.

To introduce miR-122 in the cell lines, we employed a lentiviral vector encoding pri-miR-122, an unprocessed miR-122. To confirm the maturation of pri-miR-122 to form functional RNA-induced silencing complexes (RISCs), suppression of the translation of the target mRNA was determined by a dual reporter assay. Translation of a firefly luciferase mRNA containing the sequences complementary to miR-122 in the 3′ UTR was suppressed by infection with the lentivirus encoding pri-miR-122 but not by infection with a control virus (data not shown), suggesting that the pri-miR-122 is processed into a functionally mature miR-122. By using this lentiviral vector, high levels of miR-122 expression were achieved in the 10 cell lines, comparable to the endogenous expression level of miR-122 in Huh7 cells (Fig. 4A).

To examine the effect of the exogenous expression of miR-122 on HCV replication, the nonhepatic cell lines expressing miR-122 were infected with HCVcc at a multiplicity of infection (MOI) of 1, and intracellular viral RNA was determined (Fig. 4B). The expression of miR-122 significantly increased the amount of the HCV genome in Hec1B, 293T-CLDN, MC-IXC, and RERF-LC-AI cells as well as Huh7 cells and slightly increased it in SK-OV3 and NCI-H-2030 cells. Although the levels of viral RNA in SW620, Caki-2, and SK-PN-DW cells upon expression of miR-122 were higher than those in control cells, no increase of viral RNA was observed. No effect of the expression of miR-122 was observed in 769-P cells. Interestingly, naïve Hec1B cells exhibited a delayed increase in viral RNA from 24 to 48 h postinfection, in contrast to the gradual decrease of viral RNA in other cell lines. Replication of HCV RNA in both naïve and miR-122-expressing Hec1B cells was inhibited by treatment with an inhibitor for HCV protease but not by treatment with IFN-α, due to the lack of an IFN receptor (11), whereas treatments with either IFN-α or the protease inhibitor suppressed the replication of HCV in the other cell lines expressing miR-122 (Fig. 4C). These results indicate that exogenous miR-122 expression enhances the replication of HCV even in nonhepatic cells. Hec1B cells exhibit a delayed replication of HCV, and HCV replication was enhanced by the exogenous expression of miR-122. Therefore, in this study we used Hec1B cells to investigate the biological significance of miR-122 on the replication of HCVcc in nonhepatic cells.

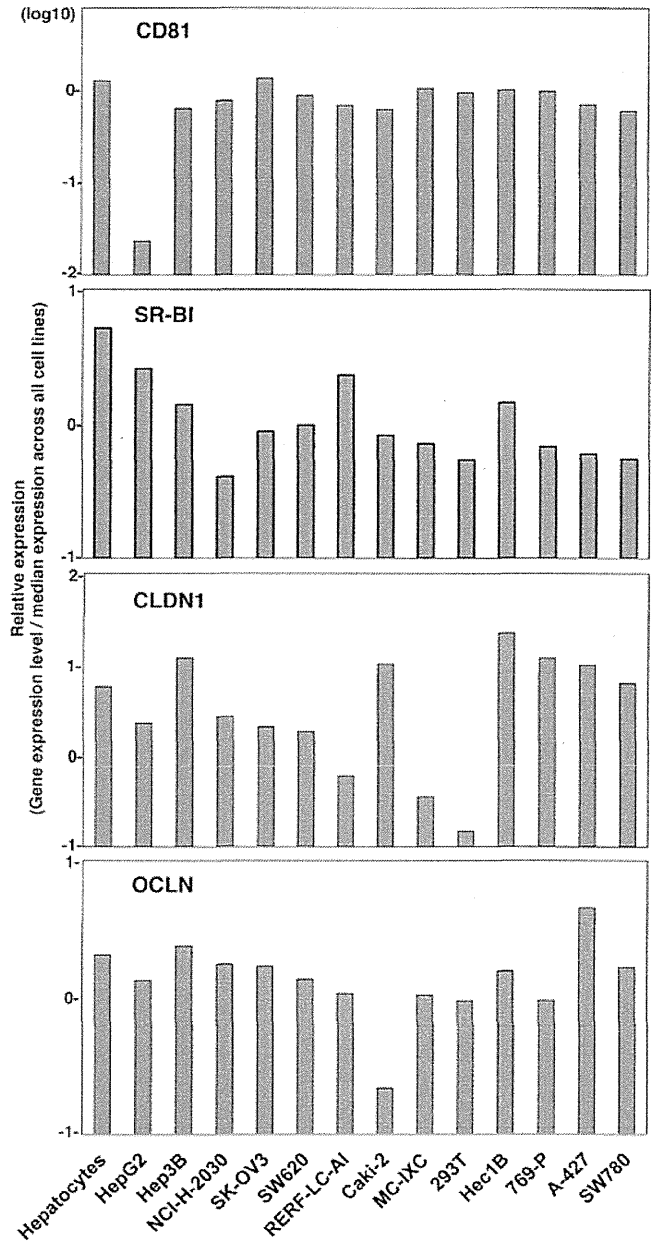

FIG 2 Receptor expression profiling in nonhepatic cells. Relative expression levels of CD81, SR-BI, CLDN1, and OCLN in primary hepatocytes, hepatic cell lines HepG2 and Hep3B, and nonhepatic cells were determined by using the NextBio Body Atlas. Expression levels were standardized by the median expression across all cell lines.

Expression of miR-122 is essential for enhancing HCV replication in Hec1B cells. To confirm the specificity of HCV replication in Hec1B cells, HCVcc was preincubated with an anti-HCV E2 monoclonal antibody, AP-33, or Hec1B/miR-122 and Hec1B/

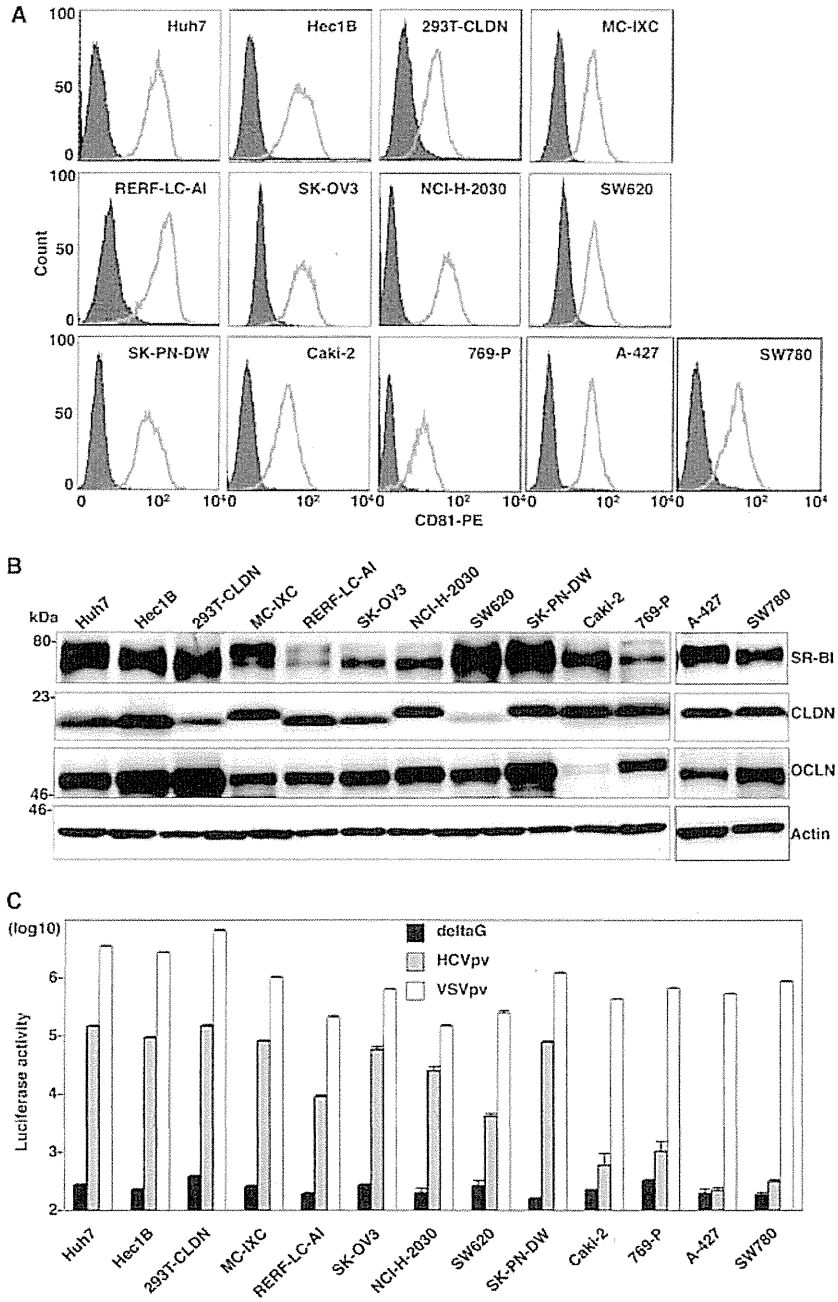

FIG 3 Expression of functional HCV receptor candidates in nonhepatic cells. (A) Expression of hCD81 in nonhepatic cells was determined by flow cytometry. PE, phycoerythrin. (B) Expression levels of SR-B1, CLDN, and OCLN in the nonhepatic cells were determined by immunoblotting. (C) The nonhepatic cell lines were inoculated with pseudotype VSVs bearing no envelope protein (deltaG), HCV envelope proteins of genotype 1b Con1 strain (HCVpv), or VSV G protein (VSVpv), and luciferase expression was determined at 24 h postinfection.

Cont cells were pretreated with anti-hCD81 monoclonal antibody. Replication of HCV RNA was determined upon infection with HCVcc. The antibody treatment significantly inhibited HCV replication in the Hec1B cell line, indicating that HCVcc internalizes into Hec1B cells through a specific interaction between hCD81 and E2 (Fig. 5). Next, we determined the dose dependence of miR-122 expression on the enhancement of HCV replication in Hec1B cells. Huh7.5.1 and Hec1B cells transduced with the lentiviral vector encoding pri-miR-122 were infected with HCVcc at an MOI of 1, and intracellular miR-122 and viral RNA were determined. Expression of miR-122 was increased in Hec1B cells in a dose-dependent manner of the lentivirus, whereas no increase was observed in Huh7.5.1 cells, probably due to the high level of endogenous expression of miR-122 (Fig. 6A, left). HCV RNA replication in Huh7.5.1 and Hec1B cells was correlated with miR-122 expression (Fig. 6A, right), suggesting a close correlation between miR-122 expression and HCV replication.

Next, we examined the expression of viral proteins in Hec1B/