thioredoxin, as well as HSP-70, in hepatocytes and other cells (15). The antiviral activity of thioredoxin is induced by AP-1 and NF-κB but not by HSP-70 (16). GGA, which has potent antiviral activities through the enhancement of antiviral factors, can clinically provide protection from influenza viral infections (17). Previously, we reported that GGA induction of antiviral proteins was dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 with which HCV was not infected (10). However, HCV products inhibit the Jak-STAT pathway in HCV-infected hepatocytes (18). The mechanism of inhibition of the Jak-STAT pathway is multifactorial and includes the expression of suppressor of cytokine signaling 3 (SOCS-3) (19), protein phosphatase 2A induction (20), STAT-3 expression (21), and IL-8 expression (22). A clarification of GGA-induced anti-HCV activity is necessary for further examination of the *in vitro* and *in vivo* effects.

The peak venous blood concentration after taking 150 mg of GGA orally is 5-7 μmol/L (23), but 50 μmol/L is the best dose for induction of PKR and 2′5′-OAS in hepatoma cell lines (10). In this study, we employed the usual dosage of GGA used to treat chronic gastritis in Japan, which is 150 mg per day. In a previous study, it was reported that portal blood concentration after taking 150 mg of GGA orally was several-fold that of the venous blood concentration (23). The usual dosage of GGA also may have a possible antiviral gene expression effect in the liver.

In conclusion, GGA, a drug that can be safely administered orally, has anti-HCV activity. Unfortunately, we did not observe a case that exhibited disappearance of HCV-RNA titers. GGA treatment is insufficient for clearance of HCV, and, therefore, it will be necessary to examine the clinical effectiveness of the combination treatment with GGA and IFN in HCV patients in the future.

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J Gastroenterol (2012) 47:195–202
DOI 10.1007/s00535-011-0481-z

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Geranylgeranylacetone has anti-hepatitis C virus activity via activation of mTOR in human hepatoma cells

Shigeyuki Takeshita · Tatsuki Ichikawa · Naota Taura · Hisamitsu Miyaaki · Toshihisa Matsuzaki · Masashi Otani · Toru Muraoka · Motohisa Akiyama · Satoshi Miuma · Eisuke Ozawa · Masanori Ikeda · Nobuyuki Kato · Hajime Isomoto · Fuminao Takeshima · Kazuhiko Nakao

Received: 27 July 2010 / Accepted: 29 August 2011 / Published online: 25 October 2011

Abstract
Background Geranylgeranylacetone (GGA), an isoprenoid compound which includes retinoids, has been used orally as an anti-ulcer drug in Japan. GGA acts as a potent inducer of anti-viral gene expression by stimulating ISGF3 formation in human hepatoma cells. This drug has few side effects and reinforces the effect of IFN when administered in combination with peg-IFN and ribavirin. This study verified the anti-HCV activity of GGA in a replicon system. In addition, mechanisms of anti-HCV activity were examined in the replicon cells.
Methods OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. After treatment, the cells were harvested with Renilla lysis reagent and then subjected to a luciferase assay according to the manufacturer's protocol.
Result The results showed that GGA had anti-HCV activity. GGA induced anti-HCV replicon activity in a time- and dose-dependent manner. GGA did not activate the tyrosine 701 and serine 727 on STAT-1, and did not induce HSP-70 in OR6 cells. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity and PKR. An additive effect was observed with a combination of IFN and GGA.
Conclusions GGA has mTOR dependent anti-HCV activity. There is a possibility that the GGA anti-HCV activity can be complimented by IFN. It will be necessary to examine the clinical effectiveness of the combination of GGA and IFN for HCV patients in the future.

Keywords mTOR · STAT-1 · Interferon · HCV · GGA

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
GGA	Geranylgeranylacetone
siRNA	Small interfering RNA

Introduction

Currently, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. The treatment of HCV with a combination of pegylated interferon (IFN) and ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. New anti-HCV agents have been developed to inhibit the life cycle of HCV and are

S. Takeshita · T. Ichikawa (✉) · N. Taura · H. Miyaaki · T. Matsuzaki · M. Otani · T. Muraoka · M. Akiyama · S. Miuma · E. Ozawa · H. Isomoto · F. Takeshima · K. Nakao
Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: ichikawa@net.nagasaki-u.ac.jp

M. Ikeda · N. Kato
Department of Molecular Biology, Graduate school of Medicine and Dentistry, Okayama University, Okayama, Japan

used in combination with IFN-α to ameliorate the salvage rate of HCV infection [2]. It is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment since IFN-α is the most basic agent for HCV treatment. Any agents that can support IFN activity will improve the therapeutic effect for HCV infected patients.

Geranylgeranylacetone (GGA), an isoprenoid compound, which includes retinoids, has been used orally as an anti-ulcer drug developed in Japan [3]. GGA protects the gastric mucosa from various types of stress without affecting gastric acid secretion [4, 5]. Moreover, GGA suppresses cell growth and induces differentiation or apoptosis in several human leukemia cells [6, 7]. Another isoprenoid compound, 3,7,11,15-tetramethyl-2,4,6,-10,14-hexadecapentaenoic acid, which is designated as an acyclic retinoid because it has the ability to interact with nuclear retinoid receptors [8], causes apoptosis in certain human hepatoma cells [9]. GGA acts as a potent inducer of antiviral gene expression by stimulating the ISGF3 formation in human hepatoma cells [10]. GGA induces the expression of antiviral proteins such as 2′5′-oligoadenylate synthetase (2′5′-OAS) and double-stranded RNA-dependent protein kinase (PKR) in hepatoma cell lines. GGA stimulates 2′5′-OAS and PKR gene expression at the transcriptional level through the formation of interferon-stimulated gene factor 3 (ISGF-3), which regulates the transcription of both genes. GGA induces the expression of signal transducers and activators of transcription 1, 2 (STAT-1, STAT-2) and p48 proteins, components of ISGF3, together with the phosphorylation of STAT1 [10]. However, no anti-HCV activity was observed.

A cell culture HCV replicon system has been developed as a useful tool for the study of HCV replication and mass screening for anti-HCV reagents. OR6 cells stably harboring the full-length genotype 1 replicon containing the *Renilla* luciferase gene, ORN/C-5B/KE [11], were used to examine the influence of the anti-HCV effect of IFN. The luciferase activity in cell lysate of OR6 was correlated with the HCV-RNA concentration, and the IC50 of IFN-α was less than 10 IU/mL [11]. The OR6 system is a useful and sensitive cell culture replicon system.

This study verified the anti-HCV activity of GGA in the OR6 system. In addition, the mechanisms of anti-HCV activity were examined in OR6 cells.

Materials and methods

Reagents

GGA was a generous gift from Eisai Co. (Tokyo, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). Wortmannin, LY294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE, were used to examine the influence of the anti-HCV effect of GGA. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fatal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication.

Reporter gene assay

The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and GGA. After treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI, USA) and luciferase activity in the cells was determined using a luciferase reporter assay system and a TD-20/20 luminometer. The data were expressed as the relative luciferase activity.

Western blotting and antibodies

Western blotting with anti-STAT-1, anti-PKR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-serine-2448 phosphorylated mTOR, anti-mTOR, anti-threonine-389 phosphorylated p70S6K, anti-p70S6K (Cell Signaling, Beverly, MA, USA) and anti-HSP70 (Stressmarq Biosciences Inc, Victoria, Canada) was performed as described previously [10]. Briefly, OR6 cells were lysed by the addition of a lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium *o*-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

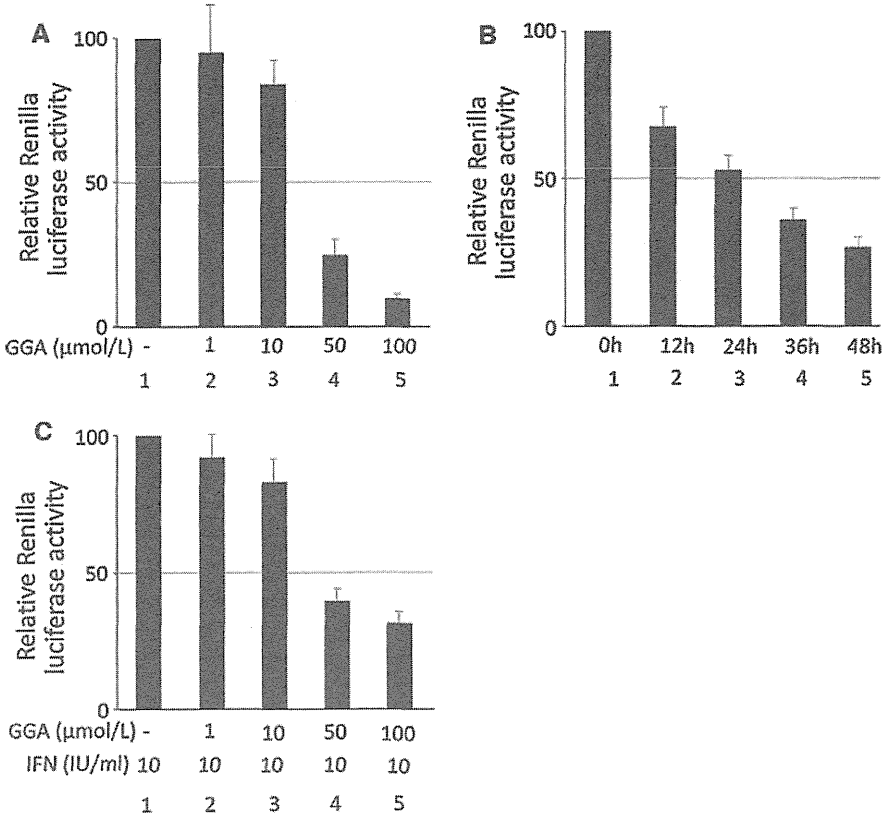

Fig. 1 The effect of GGA on the genome-length HCV RNA replication system. **a** Dose dependent effect of GGA. **b** Time course of GGA suppressed HCV replication. **c** The additive effect of GGA with IFN-α suppressed HCV replication. **a** The OR6 cells were treated with 1–100 μmol/L of GGA (*lanes 2–5*) and *lane 1* was not treated. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments. The differences between *lane 3* versus *4*, *lane 3* versus *5* and *lane 3* versus *5* were statistically significant. **b** The OR6 cells were treated 50 μmol/L of GGA and at the indicated time, HCV replicon assay was done ($n = 4$). The differences between *lane 1* versus *3–5* and *lane 2* versus *4*, *5* were statistically significant. **c** The OR6 cells were treated with 10 IU/mL of IFN-α in the absence (*lane 1*) or presence of treatment with 1–100 μmol/L of GGA (*lanes 2–5*). Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The differences between *lane 1* versus *4*, *5* were statistically significant. Statistical significance was accepted as a P value of <0.05. The data are expressed as the mean ± SD and are representative of four similar experiments

siRNA transfection assay

mTOR gene knockdown was performed using siRNA (Cell Signaling, Beverly, MA, USA). OR6 cells were transfected with100 nmol/L mTOR specific and non-targeted siRNA as a control in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 50 μmol/L GGA.

mTOR kinase activity assay

The cells were washed two times with TBS and lysed by addition of lysis buffer [50 mM Tris HCl, pH 7.4, 100 mM NaCl, 50 mM β-glycerophosphate, 10% glycerol (w/v), 1% Tween-20 detergent (w/v), 1 mM EDTA, 20 nM microcystin-LR, 25 mM NaF, and a cocktail of protease inhibitors]. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity using a commercially available kit (Calbiochem, San Diego, USA) according to the manufacturer's instructions.

Results

GGA with or without IFN had anti-HCV activity

OR6 cells, the full-length HCV replication system, were used to examine the effect of GGA. The cells were treated with 1–100 μmol/L of GGA for 24 h and the amount of HCV replicon was measured by the *Renilla* luciferase assay (Fig. 1a). The relative *Renilla* luciferase activity decreased in a dose-dependent manner. Furthermore, GGA

induced anti-HCV replicon activity was time dependent (Fig. 1b). GGA was combined with IFN-α to examine the additive effect (Fig. 1c). One or 10 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity slightly (Fig. 1c). However, 50 or 100 μmol/L of GGA combined with IFN-α decreased the relative *Renilla* luciferase activity with statistical difference. GGA treatment did not have any statistically significant effect on cell viability from 1 to 100 μmol/L of GGA for 24 h (data not shown).

GGA did not activate the tyrosine-701 and serine-727 on STAT-1, and did not induce PKR and HSP-70 in OR6 cells

GGA mediated phosphorylation of STAT-1 at the tyrosine-701 and serine-727 residues was investigated using antibodies to phospho-specific STAT-1 on OR6 cells. No phosphorylation of tyrosine-701 and serine-727 on STAT-1 was detected in OR6 cells (Fig. 2a). IFN induce anti-viral protein, PKR, and STAT-1 has an interferon stimulating responsive element (ISRE) in the promoter region [12]. The expression levels of both proteins did not change throughout this study, as indicated by a Western blotting analysis (Fig. 2b, c). Next, the role of HSP in the mechanism of GGA activity was examined because GGA is an inducer of HSP. The HSP-70 expression was increased by pre-exposure to heat shock (Fig. 2d, lanes 2, 4), but it did not increase due to the effects of GGA (Fig. 2d, lanes 3, 4).

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor, were able to cancel the GGA induced anti-HCV activity

The role of the PI3-K-Akt-mTOR pathway the anti-HCV activity of GGA was examined in OR6 cells. The cells were treated with GGA after 3 h in the presence or absence of rapamycin as an mTOR inhibitor, Akt inhibitor, or wortmannin as a PI3-K inhibitor (Fig. 3). Pretreatment with rapamycin attenuated the anti-HCV replication effect

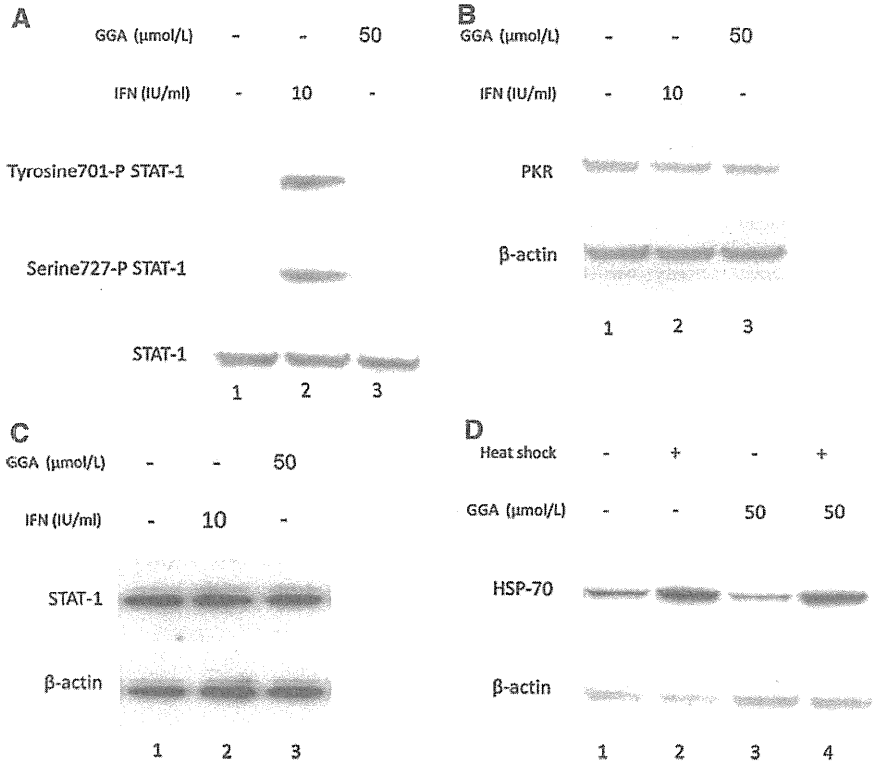

Fig. 2 Effect of GGA on STAT-1 (a), PKR (b) and HSP-70 (c). **a** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*) and then were phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and at serine-727 residue (*middle panel*), the expression STAT-1 (*lower panel*) was analyzed by Western blotting. **b** The OR6 cells were either untreated (*lane 1*) or treated with 10 IU/mL of IFN-α (*lane 2*) for 30 min or treated with 50 μmol/L GGA (*lane 3*), and then the expression of PKR (*upper panel*) was analyzed by a Western blotting analysis. The β-actin (*lower panel*) protein expression was used as an internal control. **c** The OR6 cells were either untreated (*lane 1*) or given heat shock (at 42°C 15 min, overnight recovery at 37°C) (*lanes 2, 4*) or treated with 50 μmol/L of GGA (*lanes 3, 4*) and then the expression HSP-70 (*upper panel*) was analyzed by Western blotting. β-Actin (*lower panel*) protein is the internal control

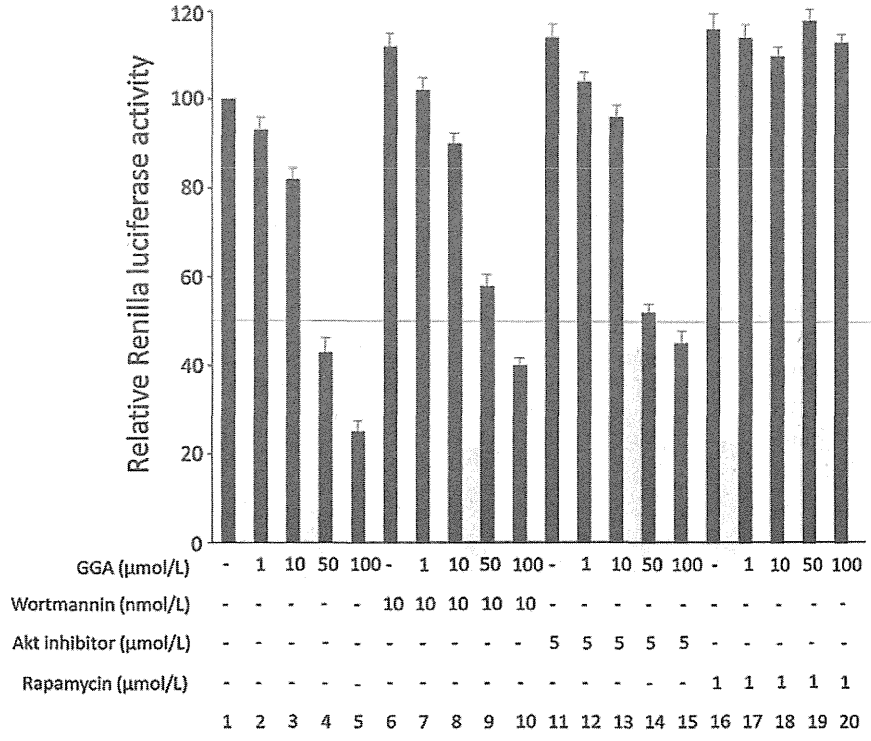

Fig. 3 Changes in GGA suppressed HCV replication by rapamycin, but not PI3-K inhibitor and Akt inhibitor. OR6 cells were treated with 1–100 μmol/L of GGA in the absence (*lanes 2–5*) or presence of pretreatment (*lanes 7–10, 12–15, 17–20*) for 3 h. *Lanes 1, 6, 11* and *16* were not treated with GGA. *Lanes 6, 11* and *16* were treated with wortmannin, an Akt inhibitor, and rapamycin, respectively. One day later, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative of four similar experiments

in comparison to GGA alone (Fig. 3, lanes 17–20), whereas pretreatment with wortmannin and Akt inhibitor did not increase the *Renilla* luciferase activity (Fig. 3, lanes 7–10, 12–15). siRNA transfection was used for mTOR knockdown to explore role of mTOR in the anti-HCV activity (Fig. 4). The transfection efficiency of the siRNA was confirmed by a Western blotting analysis. In this experiment, the detectable band intensities were quantified by the National Institutes of Health image software program. Although the transfection efficiency of siRNA was barely 46% (Fig. 4a), GGA-induced anti-HCV activity was clearly inhibited in mTOR-siRNA transfected cells (Fig. 4b, lane 4, 6) in comparison to the control cells (Fig. 4b, lanes 3, 5).

GGA induced mTOR activity, mTOR phosphorylation and p70S6K phosphorylation in OR6 cells

The phosphorylation of the serine-2448 residues of mTOR by 50 μmol/L of GGA was detected 30 min after GGA treatment. The band intensity of serine-2448 phosphorylated mTOR decreased by pretreatment with rapamycin but was almost same as with GGA alone following pretreatment with LY294002 (Fig. 5a). Furthermore, an mTOR activity assay was conducted to confirm the activity mechanism of GGA (Fig. 5b). The mTOR activity was increased by treatment with GGA alone (Fig. 5b, lane 4) and was inhibited by pretreatment with rapamycin (Fig. 5b, lane 6), whereas pretreatment with LY94002 did not suppress the mTOR activity (Fig. 5b, lane 5). Furthermore, to evaluate the mTOR activity, we investigated the level of phospholylated-p70S6K by a Western blotting analysis (Fig. 5c). The phosphorylation of the threonine-389 residue of p70S6K by 50 μmol/L of GGA was detected. Similar to mTOR, the band intensity of phospho-threonine-389 of p70S6K decreased after pretreatment with rapamycin, but the intensity was almost the same as that seen following treatment with GGA alone after pretreatment with LY294002 (Fig. 5c).

Discussion

GGA demonstrated the anti-HCV activity in this study. The anti-HCV effect depended on the GGA induced mTOR activity, not STAT-1 activity. An additive effect was observed with the combination of IFN and GGA.

GGA is a non-toxic heat shock protein (HSP) 70 inducer [13]. Various GGA activities outside of the stomach are also related to HSP induction [14–16]. GGA induced HSP-70 exerts an anti-ischemic stress activity in the heart and liver [16, 17], an anti-inflammatory activity in various cell types [18] and promotes liver regeneration [19]. GGA induces thioredoxin as well as HSP-70 in hepatocytes and other cells [20]. Thioredoxin anti-virus activity, is induced by AP-1 and NF-κB but not HSP-70 [21]. GGA has potent

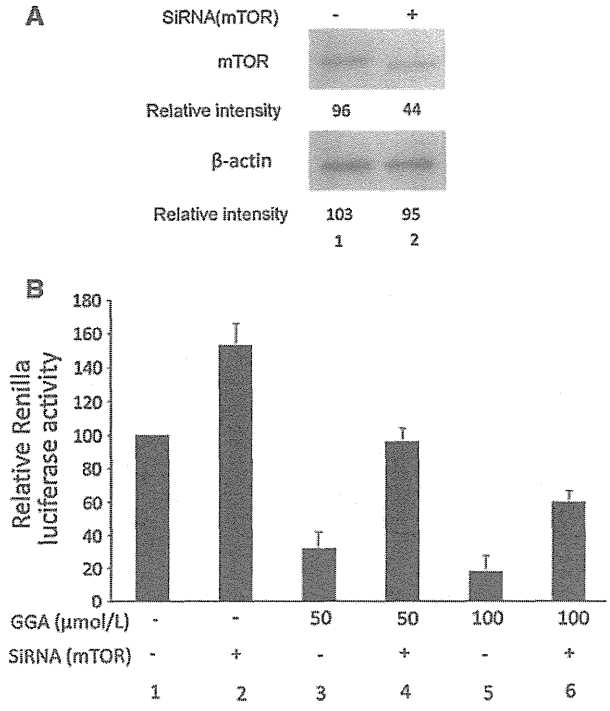

Fig. 4 Changes in GGA suppressed HCV replication by mTOR-siRNA. a OR6 cells were transfected with mTOR-siRNA (*lane 1*) or the non-targeted siRNA (*lane 2*). The expression of mTOR was evaluated by a Western blotting analysis. b The OR6 cells were transfected with mTOR-siRNA (*lanes 2*, *4* and *6*) and the non-targeted siRNA (*lanes 1*, *3* and *5*). One day later, the cells were treated with GGA (*lanes 3–6*). The HCV replicon assay is the same as Fig. 3. Non-treatment OR6 cells has 100% of relative *Renilla* luciferase light unit. The *Renilla* luciferase activity increased in the OR6 cells transfected with mTOR-siRNA (*lane 2*) in comparison to the non-targeted siRNA (*lane 1*). However, in OR6 cells treated with GGA, there was a greater elevation of *Renilla* luciferase activity in OR6 cells transfected with mTOR-siRNA (*lanes 4* and *6*) as compared to that with the non-targeted siRNA (*lanes 3* and *5*). The data are expressed as the mean ± SD and are representative example of four similar experiments

antiviral activity via the enhancement of antiviral factors and can clinically provide protection from influenza virus infection [22]. GGA significantly inhibits the synthesis of influenza virus-associated proteins and prominently enhances the expression of human myxovirus resistance 1 (MxA) followed by increased HSP-70 transcription [22]. Moreover, GGA augments the expression of an interferon-inducible double-strand RNA-activated protein kinase (PKR) gene and promotes PKR autophosphorylation and concomitantly alpha subunit of eukaryotic initiation factor 2 phosphorylation during influenza virus infection [22]. These anti-virus activities are related to GGA induced HSP-70. But, HSP-70 protein and PKR were not induced by GGA in OR6 cells in the current study. There is apparently no relationship between the GGA induced anti-HCV activity and HSP, PKR in OR6 cells. Therefore, we thought that HSP and PKR-independent anti-HCV activity induced by GGA was present in this hepatoma-derived cell line.

GGA induction of anti-viral protein is dependent upon STAT-1 tyrosine phosphorylation in HuH-7 and HepG2 [10]. However, GGA did not induce STAT-1 tyrosine phosphorylation and anti-virus protein, PKR, in OR6 cells in this study. Moreover, the GGA induced anti-HCV activity depended on mTOR activity, not STAT-1. OR6 cells are full length HCV replicon transfected HuH-7 cells [11]. HCV virus products inhibit the Jak-STAT pathway [23–25]. The mechanism of inhibition of the Jak-STAT pathway is multi-factorial including the suppressor of cytokine signaling 3 (SOCS-3) expression [26], protein phosphatase 2A (PP2A) induction [27], STAT-3 expression [28] and IL-8 expression [29]. GGA induced STAT-1 tyrosine phosphorylation and inducible PKR protein levels are also minor. Generally, the replicon transfection induces the intrinsic IFN [30], but STAT-1 tyrosine phosphorylation was not detected in combined OR6 cells. HCV replicon produced viral product might be inhibiting GGA-induced STAT-1 tyrosine phosphorylation.

mTOR is associated with the IFN induced anti-HCV signal [31]. The IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalomyocarditis virus effect [32]. IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. A relationship has been observed between the replication of the hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV [33]. The IFN induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity and Jak independent TOR activity involves STAT-1 phosphorylation and nuclear localization, and then PKR is expressed in hepatocytes [31]. No relationship between GGA and mTOR has been reported. However, GGA induced anti-HCV activity depended on mTOR activity independent of PI3-K-Akt, as observed with IFN induced mTOR activity.

When 150 mg of GGA was administered orally, the serum concentration of GGA was approximately 7 μmol/L [34]. The concentration of GGA in the portal blood would be several-fold higher than the serum concentration of GGA; therefore, we speculated that the pharmacological action that would be obtained in clinical practice would be the same as that observed in this study.

GGA, a drug that can be safely administered orally, has mTOR dependent anti-HCV activity. The combination of IFN and GGA has an additive effect on anti-HCV activity. The current results suggest that combination therapy with

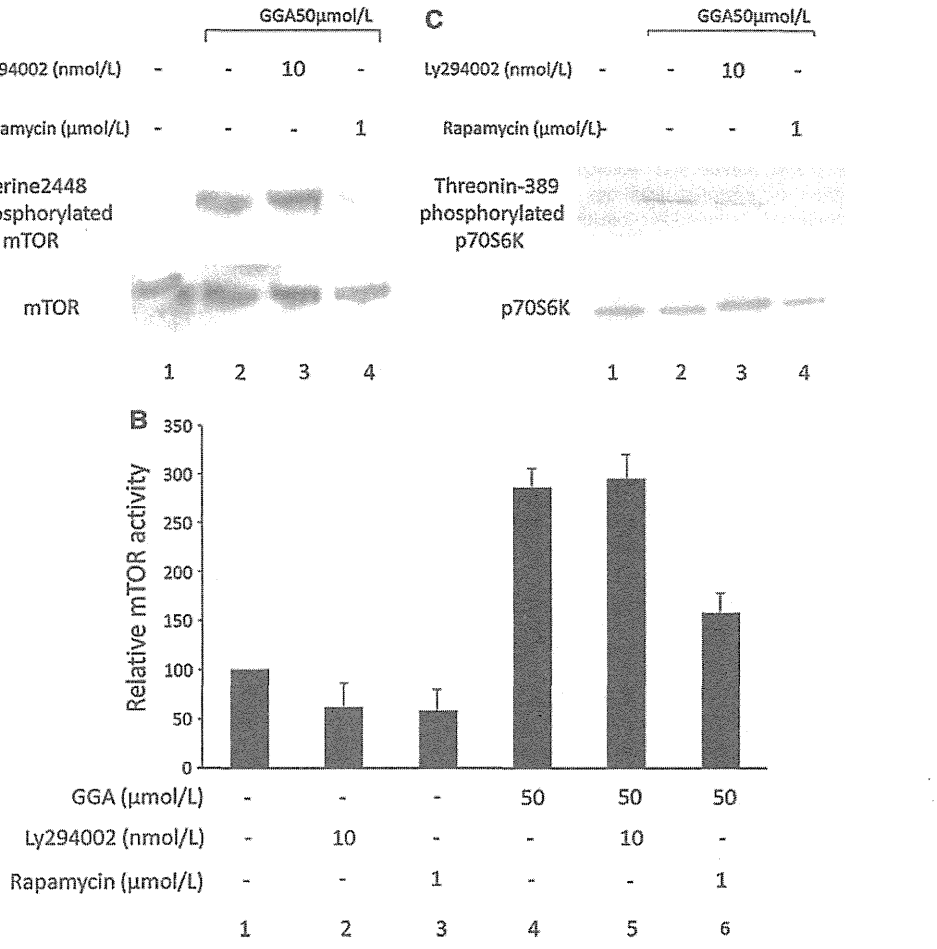

Fig. 5 Effect of GGA on mTOR and effect of LY294002 and rapamycin on GGA-induced serine phosphorylated mTOR and threonine phosphorylated p70S6K. **a** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min and then were phosphorylated mTOR at serine-2448 residue (*upper panel*), the expression of mTOR (*lower panel*) was analyzed by Western blotting. **b** After pretreatment with 10 nmol/L LY294002 (*lanes 2* and *5*) and 1 μmol/L rapamycin (*lanes 3* and *6*) for 3 h, the OR6 cells were either untreated (*lanes 1–3*) or treated with 50 μmol/L GGA (*lanes 4–6*) for 30 min. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit ($n = 4$). The differences between *lanes 1* and *4*, *lanes 4* and *6*, and *lanes 5* and *6* were statistically significant. The data are expressed as the mean ± SD and are representative of four similar experiments. **c** After pretreatment with 10 nmol/L LY294002 (*lane 3*) and 1 μmol/L, and with rapamycin (*lane 4*) for 3 h, the OR6 cells were either untreated (*lane 1*) or treated with 50 μmol/L GGA (*lanes 2–4*) for 30 min, and then were examined for phosphorylated p70S6K at the threonine-389 residue (*upper panel*), or the expression of p70S6K (*lower panel*) by a Western blotting analysis

GGA and IFN is, therefore, expected to improve the anti-HCV activity. It will, therefore, be necessary to examine the clinical effectiveness of the combination with GGA and IFN for HCV patients in the future.

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Insulin-induced mTOR activity exhibits anti-hepatitis C virus activity

TORU MURAOKA, TATSUKI ICHIKAWA, NAOTA TAURA, HISAMITSU MIYAAKI, SHIGEYUKI TAKESHITA, MOTOHISA AKIYAMA, SATOSHI MIUMA, EISUKE OZAWA, HAJIME ISOMOTO, FUMINAO TAKESHIMA and KAZUHIKO NAKAO

Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Received September 25, 2011; Accepted October 31, 2011

DOI: 10.3892/mmr.2011.662

Abstract. The mammalian target of rapamycin (mTOR) is one of the influential molecules for the anti-hepatitis C virus (HCV) action of interferon (IFN). IFN-induced mTOR activity, independent of phosphatidylinositol-3-kinase (PI3K) and Akt, is a critical factor for anti-HCV activity. mTOR activity is involved in signal transducers and activators of transcription (STAT)-1 phosphorylation and nuclear localization, and then double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes. Insulin (INS) is a major cytokine for metabolism and regulates the PI3K-Akt-mTOR signaling pathway in hepatocytes. Changes in mTOR activity have been reported in chronic HCV-infected patients with excess nutrition and INS resistance. Therefore, this experiment investigated whether INS increases anti-HCV activity via mTOR activity. This study used a genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), derived from HuH7 cells. OR6 cells were pre-treated with rapamycin or LY294002 or siRNA, and the cells were treated with INS (0-300 nmol/l) or IFN (0-50 IU/ml) for 30 min to 48 h. The cells were lysed and analyses were carried out using the *Renilla* luciferase assay, western blotting or ELISA. INS induced the anti-HCV effects via mTOR activity, independently of STAT-1 tyrosine phosphorylation, in a dose- and time-dependent manner. INS-induced mTOR activation was found to be PI3K-Akt-dependent in OR6 cells. The combination of IFN and INS had an additive anti-HCV effect. The INS-induced mTOR activity was identified to be an anti-HCV signal independent of the STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should, therefore, attempt to identify new agents that activate mTOR to promote anti-HCV activity.

Correspondence to: Dr Toru Muraoka, Department of Gastroenterology and Hepatology, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
E-mail: tourumura@hotmail.com

Abbreviations: IFN, interferon; INS, insulin; HCV, hepatitis C virus; CHC, chronic hepatitis C; Jak, Janus kinase; STAT, signal transducers and activators of transcription; ISRE, IFN-stimulated regulatory element; PKR, double-stranded RNA-dependent protein kinase; Rapa, rapamycin; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA

Key words: mammalian target of rapamycin, signal transducers and activators of transcription-1, interferon, hepatitis C virus, insulin

Introduction

At present, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide (1). Although HCV is a hepatotropic virus, chronic HCV infection causes many metabolic disorders, including diabetes (2), insulin resistance (3) and hepatic steatosis (4). These metabolic disorders are related to an interferon (IFN) refractory condition (5-7). Treatment of HCV using a combination of pegylated IFN and ribavirin is effective in less than 50% of chronic hepatitis C (CHC) patients with genotype 1 virus (8). New anti-HCV agents, such as protease (9), polymerase (10) and cyclophilin inhibitors (11), have been developed to inhibit the HCV life cycle, but single application of these newly developed drugs is not effective enough for HCV eradication. Since IFN-α is the most basic agent for HCV treatment, it is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The most important intra-hepatocellular signal transduction of the IFN pathways is the Janus kinase (Jak)-signal transducers and activators of transcription (STAT) signal (12). Mammalian target of rapamycin (mTOR) is one of the influential molecules associated with IFN-induced anti-HCV action (13). The IFN-activated mTOR pathway plays an important regulatory role in the promotion of the IFN effect, including the anti-encephalomyocarditis virus effect (14). IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE)-dependent promoter gene activity. Human cytomegalovirus is inhibited by AMP-activated protein kinase-mediated inhibition of mTOR kinase (15). By contrast, vesicular stomatitis virus is mTOR-dependent (16). A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70

S6 kinase pathway and regulates the replication of HCV (17). IFN-induced mTOR activity, independent of PI3K and Akt, is a critical factor for the anti-HCV activity, and Jak-independent mTOR activity involves STAT-1 phosphorylation and nuclear localization; subsequently, double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes (13).

mTOR activity has pleiotrophic functions, such as cell growth (18), nutrition control (19) and immunoregulation (20). However, there has been little examination of the influence that mTOR activity has on HCV proliferation. mTOR activity does not influence HCV-ISRE activity as the viral promoter has cap-independent translation (21). Although mTOR is a mRNA translational regulator that acts through phosphorylation of a downstream target such as 4E-BP and S6K (22), IFN-induced mTOR activity influences the phosphorylation of STAT-1 (13). Changes in mTOR activity in CHC patients with extra nutrition or with insulin resistance have been reported (23). However, the anti-HCV effect associated with mTOR activity remains to be sufficiently elucidated.

Insulin (INS) is a major cytokine for metabolism and acts via the PI3K-Akt-mTOR signaling pathway in hepatocytes (24). INS is indirectly related to HCV-associated INS resistance. Therefore, this study investigated whether INS increases the anti-HCV activity via mTOR activity.

Materials and methods

Reagents. INS was purchased from Eli Lilly Japan (Kobe, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). LY294002 and rapamycin (Rapa) were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system. OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE (25), were used to examine the influence of the anti-HCV effect of IFN and INS. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and maintained in the presence of G418 (300 mg/l; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI, USA) and then subjected to a luciferase assay according to the manufacturer's protocol. The data were expressed as the relative luciferase activity.

Reporter gene assay. The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers, IFN and INS, and the luciferase activities in the cells were determined using a luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative luciferase activity.

Cell viability assay. The cells were assessed using a colorimetric cell viability assay method. Cell viability was determined by a colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). Cell viability after 2 days of 100 IU/ml of IFN-α and 1000 nmol/l of Rapa treatment was expressed as a percentage of the viability in standard media without IFN-α and Rapa. Statistical significance was assessed using the Student's t-test, and a difference was considered to be statistically significant at $P<0.05$.

Western blotting and antibodies. Western blotting with anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-mTOR and anti-serine-2448 phosphorylated mTOR (Cell Signaling, Beverly, MA, USA) was performed as described later. OR6 cells were lysed by the addition of a lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mmol/l sodium o-vanadate and 1 mmol/l NaF). The samples were separated by electrophoresis on 8-12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling), and the immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

mTOR kinase assay. The K-LISA mTOR Activity kit (Calbiochem) was used in this study. OR6 cells were incubated in the absence or presence of INS for 30 min with or without pre-treatment with Rapa for 12 h. The cells were washed two times with TBS and lysed by addition of a lysis buffer that was recommended by the manufacturer. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4°C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity according to the manufacturer's protocol. The absorbance was measured with a Multiskan JX microplate reader.

siRNA transfection assay. Knockdown of the mTOR gene was performed using siRNA (Cell Signaling), and 100 nmol/l mTOR-specific and non-targeted siRNA as a control was used to transfect OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/ml IFN or 300 nmol/l INS.

Results

INS exhibits anti-HCV activity with or without IFN. OR6 cells, a full-length HCV replication system, were used to examine the anti-viral effect of INS and IFN. The cells were incubated in medium that contained 0-100 nM of INS and 0-50 IU/ml of IFN for 48 h, and were harvested for a *Renilla* luciferase assay (Fig. 1). The relative *Renilla* luciferase activity decreased in an INS dose-dependent manner when the IFN concentration was 0 or 1 U/l. Cell viability in the presence of INS showed no statistically significant difference with or without IFN treatment for 24 or 48 h (data not shown). Rapa and LY294002 also showed no significant difference in comparison to the control (data not shown).

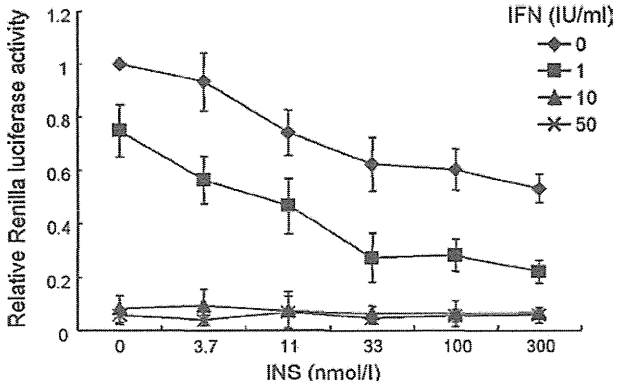

Figure 1. Effect of INS on HCV replication with or without IFN-α. OR6 cells were treated with 0-100 nmol/l INS and 0-50 IU/ml IFN. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). The data are expressed as the means ± SD and are a representative example of four similar experiments.

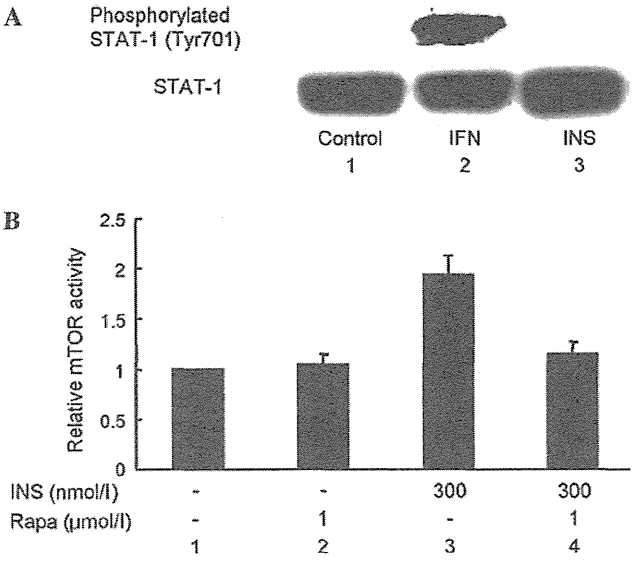

Figure 2. INS does not induce tyrosine phosphorylation of STAT-1 but induces mTOR kinase activity. (A) OR6 cells were incubated in medium only (control), and with IFN or INS for 30 min. Phosphorylation of STAT-1 at tyrosine-701 residue was analyzed by western blotting. (B) OR6 cells were not pre-treated (bars 1 and 3) or pre-treated with 1 μM Rapa (bars 2 and 4) for 12 h, and the cells were treated with 300 nM of INS (bars 3 and 4) for 30 min. Bars 1 and 2, cells not INS-treated. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 3 vs. bars 1, 2 and 4 by the Student's t-test (P<0.05).

INS does not activate tyrosine on STAT-1, but induces mTOR kinase activity. OR6 cells were incubated in medium only or with 50 IU/ml of IFN or 300 nmol/l of INS for 30 min, and were lysed for western blotting (Fig. 2A). INS did not induce phosphorylation of Tyr 701 of STAT-1 (Fig. 2A, lane 3). IFN-induced anti-viral protein PKR was also not detected by western blotting under the same condition (data not shown). Treatment with 300 nmol/l of INS induced mTOR activity in OR6 cells. INS-induced mTOR kinase activity was suppressed in OR6 cells pre-treated with Rapa for 12 h before INS stimulation (Fig. 2B, bar 4).

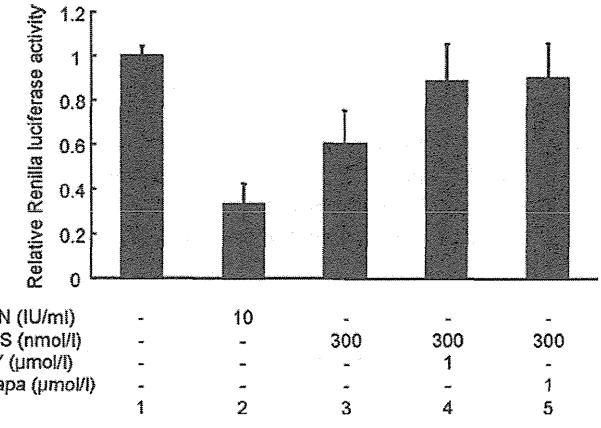

Figure 3. Changes in INS suppress HCV replication by PI3K and mTOR. OR6 cells were treated with 10 IU/ml IFN (bar 2) or with 300 nmol/l INS (bars 3-5) in the absence (bar 3) or presence of pre-treatment (bars 4 and 5) with LY294002 (LY) or rapamycin (Rapa) for 12 h. Bar 1, not treated. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). Data are expressed as the means ± SD and are a representative example of four similar experiments. A statistically significant difference was observed between bar 3 vs. bars 1, 4 and 5 by the Student's t-test (P<0.05).

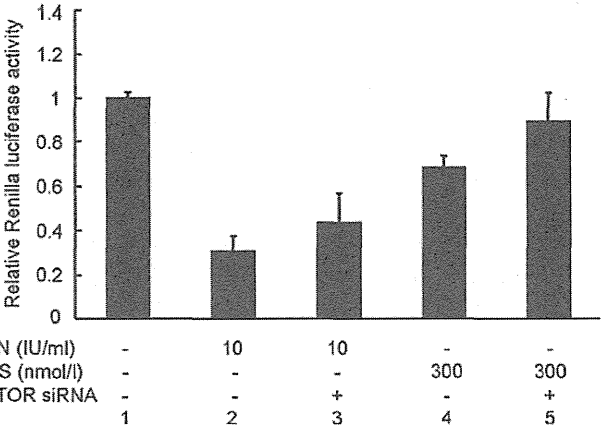

Figure 4. Changes in INS suppress HCV replication by siRNA against mTOR. OR6 cells were transfected by siRNA against mTOR (bars 3 and 5) or vehicle siRNA (bars 1, 2 and 4). One day later, the cells were treated with IFN (bars 2 and 3) or INS (bars 4 and 5). Thereafter, 1 day later, the *Renilla* luciferase activity was determined (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 4 vs. bars 1 and 5 by the Student's t-test (P<0.05).

INS-induced anti-HCV activity is inhibited by blunted mTOR activity. The role of the PI3K-Akt-mTOR pathway in INS-induced anti-HCV activity was examined in OR6 cells. The cells were treated with INS after 12 h in the presence or absence of Rapa as an mTOR inhibitor, or LY294002 as a PI3K inhibitor (Fig. 3). Pre-treatment with LY294002 or Rapa attenuated the anti-HCV effect in comparison to INS alone. siRNA transfection for mTOR knockdown was used to explore the role of mTOR activity in INS-induced anti-HCV activity (Fig. 4). IFN-induced anti-HCV activity dependent on mTOR was inhibited by mTOR siRNA. Although the transfection efficiency of siRNA was barely 10%, INS-induced anti-HCV activity was clearly inhibited in mTOR siRNA-transfected cells (Fig. 4, bar 5).

Discussion

INS-induced mTOR activity exhibits anti-HCV action independently of STAT-1 phosphorylation. mTOR activated by INS was PI3K-Akt-dependent in hepatocytes. Notably, The STAT-1-independent anti-HCV activity did not induce the IFN-induced anti-viral protein PKR.

The mechanism of INS-induced anti-HCV activity is different from that of IFN. IFN-induced anti-HCV activity depends on IFN-induced anti-viral protein via Jak-STAT signaling (12). A genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), which is an effective screening tool (25) has been used to identify a more effective therapy, especially for CHC patients with genotype 1. The OR6 system demonstrated the IFN-independent anti-HCV activity of statins (26). Another replicon system was used to demonstrate the IFN-independent anti-HCV activity of cyclosporine A (27). Both drugs have been associated with the life cycle of HCV and have an inhibitory effect on HCV replication (28,29). INS-induced anti-HCV activity may be associated with the life cycle of HCV, but not IFN-induced anti-virus protein. The susceptible point in the HCV life cycle is uncertain, but mTOR activity is an important factor which contributes to the inhibition of HCV proliferation.

Various relationships have been reported between mTOR activity and insulin resistance. mTOR was found to play a key role in IL-6-induced hepatic insulin resistance by regulating STAT3 activation and subsequent SOCS3 expression in an IL-6-induced insulin resistance model (30). Unsaturated fatty acids were found to inhibit PTEN expression in hepatocytes, leading to hepatic steatosis, insulin resistance, inflammation and cancer, by up-regulating microRNA-21 synthesis via an mTOR/NF-κB-dependent mechanism (31). mTOR activity was found to induce IRS serine phosphorylation leading to IRS degradation resulting in insulin resistance (23). HCV infection of hepatocytes stimulates insulin resistance through multifactors, including IRS degradation (32) and SOCS-3 expression (33). The present study of the association of mTOR activity and HCV proliferation suggests that insulin resistance is a condition that suppresses excessive proliferation of HCV and may be associated with chronic HCV infection.

INS-induced anti-HCV activity may be dependent on the PI3K-Akt-mTOR pathway, and one aspect of IFN-induced anti-HCV activity depends on mTOR activity (13). In this study, the combination of IFN and INS had an additive anti-HCV effect. Amino acids and INS have been demonstrated to have an additive effect on mTOR activity, since these effectors use different pathways to induce mTOR activity (34). Efficient anti-HCV activity via mTOR activity requires a combination of effectors that operate via different mTOR activation pathways. mTOR can be activated via three different pathways. The IRS-PI3K-Akt-mTOR pathway is stimulated by extracellular effectors, including INS and INS-like growth hormone (35). Activated mTOR inhibits the IRS function and generates negative feedback for IRS signaling (36). There is also nutrition-associated Akt-independent and wartmannin-dependent, or Akt-independent and adenosine monophosphate kinase-dependent mTOR activation. Amino acids and glucose inhibit Akt-dependent glycolysis through mTOR activation (37). Future studies should investigate agents that enhance IFN-induced Akt-independent mTOR activity via other mTOR-activated pathways.

INS-induced mTOR activity was found to be an anti-HCV signal-independent STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should therefore identify new anti-HCV agents that activate mTOR activity.

Acknowledgements

The authors thank Masanori Ikeda and Nobuyuki Kato of the Department of Molecular Biology, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan, for the OR6 cells.

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Kamihira et al. Virology Journal 2012, 9:40
http://www.virologyj.com/content/9/1/40

VIROLOGY JOURNAL

RESEARCH Open Access

Paradoxical expression of *IL-28B* mRNA in peripheral blood in human T-cell leukemia virus Type-1 mono-infection and co-infection with hepatitis C Virus

Shimeru Kamihira[1*], Tetsuya Usui[2], Tatsuki Ichikawa[3], Naoki Uno[1,2], Yoshitomo Morinaga[1,2], Sayaka Mori[2], Kazuhiro Nagai[2], Daisuke Sasaki[1,2], Hiroo Hasegawa[1,2], Katsunori Yanagihara[1,2], Takuya Honda[3], Yasuaki Yamada[1,2], Masako Iwanaga[4], Takashi Kanematu[5] and Kazuhiko Nakao[3]

Abstract

Background: Human T-cell leukemia virus type-1 (HTLV-1) carriers co-infected with and hepatitis C virus (HCV) have been known to be at higher risk of their related diseases than mono-infected individuals. The recent studies clarified that IL-28B polymorphism rs8099917 is associated with not only the HCV therapeutic response by IFN, but also innate immunity and antiviral activity. The aim of our research was to clarify study whether IL-28B gene polymorphism (rs8099917) is associated with HTLV-1/HCV co-infection.

Results: The genotyping and viral-serological analysis for 340 individuals showed that IL-28B genotype distribution of rs8099917 SNP did not differ significantly by respective viral infection status. However, the IL-28B mRNA expression level was 3.8 fold higher in HTLV-1 mono-infection than HTLV-1/HCV co-infection. The high expression level was associated with TT (OR, 6.25), whiles the low expression was associated with co-infection of the two viruses (OR, 9.5). However, there was no association between down-regulation and ATL development (OR, 0.8).

Conclusion: HTLV-1 mono-infection up-regulates the expression of IL-28B transcripts in genotype-dependent manner, whiles HTLV-1/HCV co-infection down-regulates regardless of ATL development.

Keywords: IL-28B, IL-λ3, HTLV-1, HCV, SNP

Introduction

A retrovirus, human T-cell leukemia virus type-1 (HTLV-1), and a positive-strand RNA virus, hepatitis C virus (HCV), are completely different in terms of virologic characteristics. Nevertheless, they play a similar role in the pathogenesis of viral-induced malignant neoplasms, such as adult T-cell leukemia (ATL) in HTLV-1- infected individuals, and hepatocellular carcinoma (HCC) and B-cell lymphoma in HCV- infected individuals, during long-term chronic infections.

Furthermore, it is known that co-infection with HCV and HTLV-1 is frequently observed in an area endemic for HTLV-1. HCV/HTLV-1 co-infected individuals have been reported to be at higher risk for developing HCC than those infected with HCV alone [1-3]. Although the pathologic mechanism of the co-infection remains to be elucidated, it is thought that the impaired immunity due to HTLV-1 infection may contribute to HCV infection and HCV-related disorders, which is suggested by previous reports. Kohno et al. reported that the severe immunodeficiency and anergic state in patients with ATL may be associated with a functional property of leukemic cells originating from regulatory T-cells expressing CD4, CD25, CCR4, GITR and Foxp3 [4]. Kishihara et al. also reported that impairment of the immune response by HTLV-1 could explain the reduced effectiveness of interferon (IFN) treatment in patients co-infected with HTLV-1 and HCV [5].

* Correspondence: kamihira@nagasaki-u.ac.jp
[1]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, 852-8501, Japan
Full list of author information is available at the end of the article

Recently, genome-wide association studies of patients with HCV have made great advances in viral clearance associated with *IL-28B* single nucleotide polymorphisms (SNP) [6,7]. IL-28B is a type III Lambda interferon (IFN-λ) and a cytokine similar to IL-10 with IFN-like activities [8]. This new IFN-λ family includes IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) [9]. Although the IFN-λ genomic structure resembles that of the IL-10 family [10], the amino acid and functional level of IFN-λs are more closely related to type I IFNs than IL-10. The IFN-λs are induced by stimulation with several single-strand RNAs (ssRNA) and several kinds of viruses. The IL-28B SNPs, such as rs8099917, rs12979860, and 12980275, have been reported to be associated with spontaneous clearance [10], innate HCV immunity [9], HCV-related disease chronicity, and therapeutic response to pegIFN-α and ribavirin (RBV) [6,7].

From these observations, we hypothesized that IFN-λ3 encoded from the *IL-28B* gene would be associated with HTLV-1 infection. The aim of the present study was to examine the mutual association between IL-28B polymorphism (rs8099917 SNP) and mono-infected-HTLV-1 and co-infected HTLV-1 with HCV subjects.

Materials and methods

Clinical subjects

All subjects were of Japanese origin living in Nagasaki City, an endemic area for HTLV-1 in Japan. For genomic specimens, 340 blood samples were randomly collected from patients who visited a liver clinic and liver transplantation center from April 2009 to March 2011 from the departments of Hepatology and a Hematology Clinic. One hundred and twenty-four of the 340 samples were also available for total RNA tests. Accordingly, most patients had either chronic liver disease (CLD) or adult T-cell leukemia (ATL). This study was done under informed consent after the approval of the Nagasaki University hospital IRB (IRB Approval No.10050). Since the samples used here were un-linked materials, patient information was restricted.

Cell lines

Eight HTLV-1-infected T-cell lines, Hut 102, MT-1, MT-2, ST1, KK1, KOB, OMT, and LMY-1, were used for IL-28B mRNA quantification. The first three were purchased and latter five were established in our laboratory [11].

Serological and genetic tests for HCV and HTLV-1

HCV and HTLV-1 infections were mainly serologically detected using commercially available kits, CLEIA-anti-HTLV-1, Lumipulse-II Ortho HCV (Fujirebio-INC, Tokyo, Japan). The confirming examination was genetically performed by the Cobas TaqMan HCV test (TaqMan HCV; Roche Tokyo INC, Tokyo, Japan) for HCV and in-house HTLV-1 proviral real-time RT quantifiable PCR [12]. Genomic DNA and total RNA were extracted from peripheral blood mononuclear cells (PBMC) using commercially available QuickGene DNA Whole blood kits (FUJIFILM Corp., Tokyo, Japan) and PureLink RNA Micro Kits (Invitrogen Corp., Carlsbad, Ca, USA). The extraction protocol was performed according to the manufacturer's instructions.

Genotyping for SNPs

SNP genotyping was performed using multiplex PCR amplification and Pyrosequencing technology. To amplify target regions, newly designed biotinylated-primers were employed: sense and anti-sense for rs8099917, 5'-TCCTCCTTTTGTTTTCCTTTCTG-3' and 5'-AAAAAGCCAGCTACCAAACTGT-3'. Then, the amplicon was sequenced according to the manufacturer's instructions based on Pyrosequence technology (Qiagen, Hilden, Germany). Biotin-labeled amplicons from the 1st PCR were captured by binding to streptavidin-coated Sepharose beads, and DNA was denatured to produce an ssDNA template for the Pyrosequencing assay. The ssDNA was released and combined with the sequencing primer, which was extended during the Pyrosequencing reaction to provide the sequence of the template DNA. Pyrosequencing data were produced in the form of Pyrograms, and genotypes were assigned by the peak pattern presented in the Pyrogram.

Real-time reverse transcription (RT) quantifiable PCR for IL-28B mRNA

mRNA for IL-28B transcribed into cDNA by a GoScript™ RT System (Promega, Madison, WI, USA) was quantified by a LightCycler System (Rosche, Mannheim, Germany) using newly designed sense and anti-sense primers, 5'-AAGGACTGCAAGTGCCGCT-3' and 5'-GCTGGTCCAAGACATCCC-3' (AY129149). A standard curve was generated using a tenfold dilution method with a reference material derived from pTAC-1.2735 inserted with 166 base fragments including the target. The amplicon was assayed by the Cyber green method. The raw data were normalized by *abl* mRNA density and evaluated as the relative value for *abl* gene expression calculated by *IL-28B* data/*abl* data $\times 10^4$, modified from our previous mRNA real time RT qPCR method [12].

Statistical analysis

The minor-allele frequency (MiAF) was set as the less frequent allele in a population for SNPs analyzed. Viral infectious status was divided into 4 groups of HTLV-1 mono-infection, HCV mono-infection, HTLV-1/HCV-co-infection, and non-infection (double negative; DN).

Differences in the genotype distribution of IL-28B SNPs among groups were compared using the Chi-square or Fisher exact test. The level of mRNA expression was compared using the Mann Whitney U test. Correlation analysis was performed by the Nonparametric Spearman's rank correlation method. The relationship between a factor and an outcome was estimated the magnitude of the association by the odds ratio with 95% confidence intervals (95%CI). Statistical analysis was performed using SAS 9.1. The statistical significance level was set at 0.05.

Results

IL-28B genotypes and the sero-status

Three hundred and forty samples were genotyped on IL-28B rs8099917 SNP and were serologically examined for viral infection of HTLV-1 and HCV. As summarized in Table 1. They consisted of 263 (77.4%) major TT homozygotes, 171 (20.9%) minor TG heterozygotes, and 6 (1.8%) minor GG homozygotes. The virus tests revealed that 59 were negative for both HTLV-1 and HCV, 73 were positive for HTLV-1 alone, 179 were positive for HCV alone and 29 were positive for both viruses. The genotypic distributions, as well as minor allele frequency (MAF) of the IL-28B gene, did not significantly differ among each viral infection status as a control of no-infection.

Since the HTLV-1 mono-infection group consisted of 47 ATL patients and 26 HTLV-1 carriers, we stratified the two groups of ATL patients and carriers and the minor allele frequencies of the two groups were compared; the difference between that of ATL and carriers was not statistically significant (p = 0.21). The prevalence of TT was not different statistically either (p = .495).

Next, the expression levels of IL-28B were quantified using 124 samples randomly collected during this study period.

Table 1 IL-28B genetic distribution and allele frequency in stratification based on the combination of HTLV-1 and HCV infection

		Genotype r(rs8099917)			Allele fequency		
	No	TT	TG	GG	T	G	
All cases	340	263 (77.4%)	71 (20.9%)	6 (1.8%)	0.86	0.14	
1) non-Infection	59	45(76.3)	10 (16.9)	4(6.8)	0.84	0.15	
2) HTLV-1 mono	73	55(75.3)	17 (23.3)	1(1.9)	0.87	0.13	P = 0.90
ATL patients	47	37(78.7)	10 (21.3)	0(0.0)	0.89	0.11	P = 0.11
carriers	26	18(69.2)	7(26.9)	1(3.8)	0.82	0.18	P = 0.46
3) HCV-mmono	179	141 (78.7)	37 (20.7)	1(1.0)	0.89	0.11	P = 0.68
4) co-infection	29	22(75.9)	7(24.1)	0(0.0)	0.88	0.14	P = 0.9

There was no significant difference in the genetic distribution and allele frequency among respective infectious states

P values were compared with non infection

IL-28B mRNA expression level and HCV/HTLV-1 co-infection

The expression levels of IL-28B mRNA were generally low in most cases with a median value of 5.2 in no-infection, 10.6 in HTLV-1 mono-infection, 3.9 in HCV mono-infection, and 2.8 in HTLV-1/HCV co-infection (Figure 1a). Notably, a small number of measurement values shown as open circles was high, and they were distributed only within the HTLV-1 mono-infected and HCV mono-infected groups. Moreover, all of those who had high values were exclusively TT homozygous, as shown in Figure 1b (samples marked by $^{A\text{-}E)}$ were the same in Figure 1(a) and Figure 1(b)). Surprisingly, the median value was the highest in HTLV-1 mono-infection and the lowest in the co-infection group (10.6 versus 2.8; p = 0.013). Therefore, to clarify whether ATL cells directly affect the expression of IL-28B mRNA, we compared the mRNA expression levels in mainly HTLV-1 carriers, ATL patients with ATL cells, and ATL cell lines. As shown in Figure 2, the median values were significantly higher in mono HTLV-1 carriers with TT (17.9 vs 5.6, $P < 0.05$) and ATL patients with TT having ATL cells than those of non-infected individuals (13.4 vs 5.6, $p < 0.05$). No high expression level was observed in two ATL or 16 carriers with HTLV-1/HCV co-infection. Surprisingly, these data were lower rather those from TG/GG. On the other hand, IL-28B mRNA expression in 8 HTLV-1-infected T-cell lines was undetectable in all but one (Hut 102). The genotype was TT in all cell lines.

In addition, there was no correlation between the IL-28B mRNA levels and HCV-RNA levels (non-parametric Spearman's rank correlation, R^2 = 0.0543, Figure 3).

Assessment by odds ratio analysis for an outcome if a risk factor is present

As shown in Figure 2, HTLV-1 was revealed to have an association similar to HCV and IL-28B mRNA. However, the up-regulated-action of HTLV-1 was nullified if the virus was co-infected with HCV. The prevalence of a major TT and minor TG/GG was similar among individuals infected with either HTLV-1 or HCV, as well as the allele frequency, indicating that there is no specific correlation between IL-28B and HTLV-1. Thus, to approach a causative clue, assessment by odds ratio (OR) analysis was performed (Table 2). Only the high mRNA level besides 3 states of HTLV-1 mono-infection,

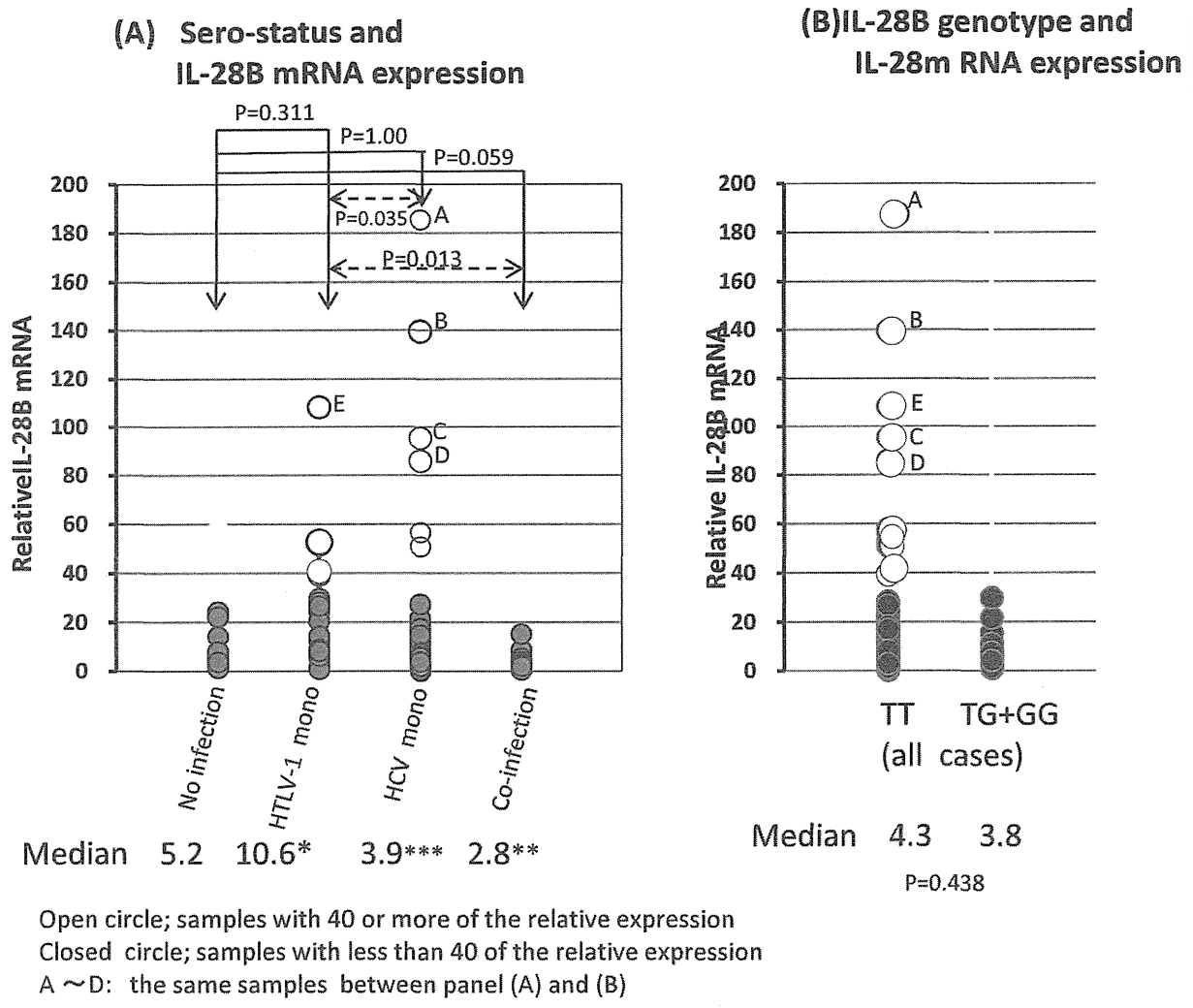

Figure 1 Relative individual IL-28B mRNA levels in peripheral blood mononuclear cells among viral infectious groups (a) and among the TT and TG/GG genotypes (b). The median values were significantly different between * vs ** and *** vs **. Open circles; those with high IL28BmRNA with TT homozygotes.

co-infection with HCV and ATL was associated with TT genotype (OR = 6.25). On the other hand, down-regulation of the mRNA density was defined as HTLV-1/HCV co-infection (OR = 9.5 p = 0.004), but low expression was not associated with ATL development (OR = 0.8, p = 0.81).

Discussion

Although co-infection with HTLV-1 and HCV has been shown to result in higher rates of cirrhosis and increased death from liver diseases [1,2], the caustic mechanism by which the co-infection affects HCV pathogenesis remains to be elucidated. Some clue to the mechanism may be found by studying the relation between IL-28B genotypes and co-infection, because IL-28B encoding IFN-λs are categorized as type 3 IFNs and are potent endogenous anti-viral cytokines. They signal via JAK/STAT intracellular pathways and up-regulated transcription of IFN-stimulated genes (ISGs) that are required to control viral infection [13]. Here, we investigated whether IL-28B polymorphism rs8099917 is associated with co-infection status.

The present study is the first to reveal that the IL-28B genotype is not associated with stratification based on the combination of HTLV-1 and HCV infection; no infection for both (double negative; DN), HTLV-1 mono-infection, HCV mono-infection and HTLV-1/HCV co-infection. Similarly, the frequency of the major TT homozygotes was not associated among ATL patients and HTLV-1 carriers (Table 2). These two

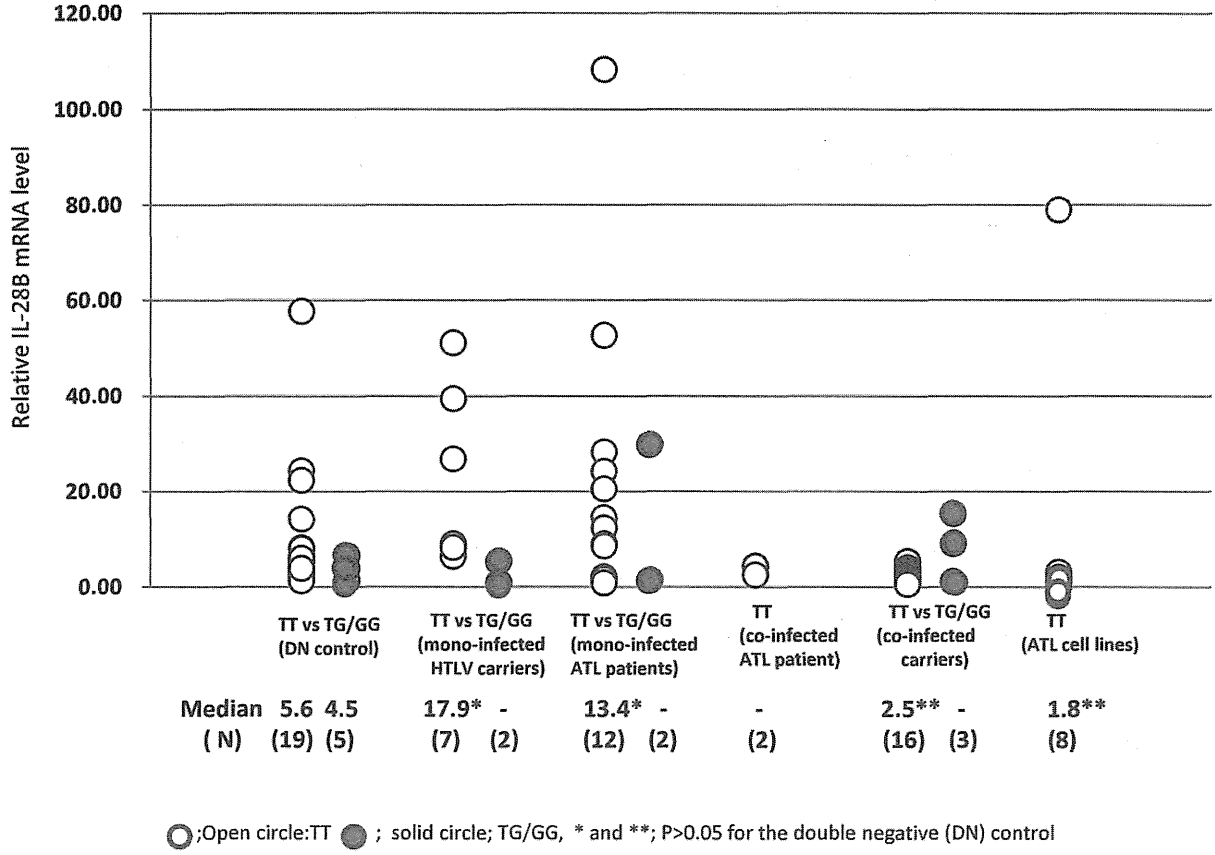

Figure 2 Individual relative expression density of IL-28B mRNA in TT vs TG/GG derived from 24 double-negative (DN) controls consisting of 19TT and 5TG/GG, 9 mono- infected HTLV-1 carriers, 14 mono-infected and 2 co-infected ATL patients, 19 co-infected carriers and 8 ATL cell lines samples.

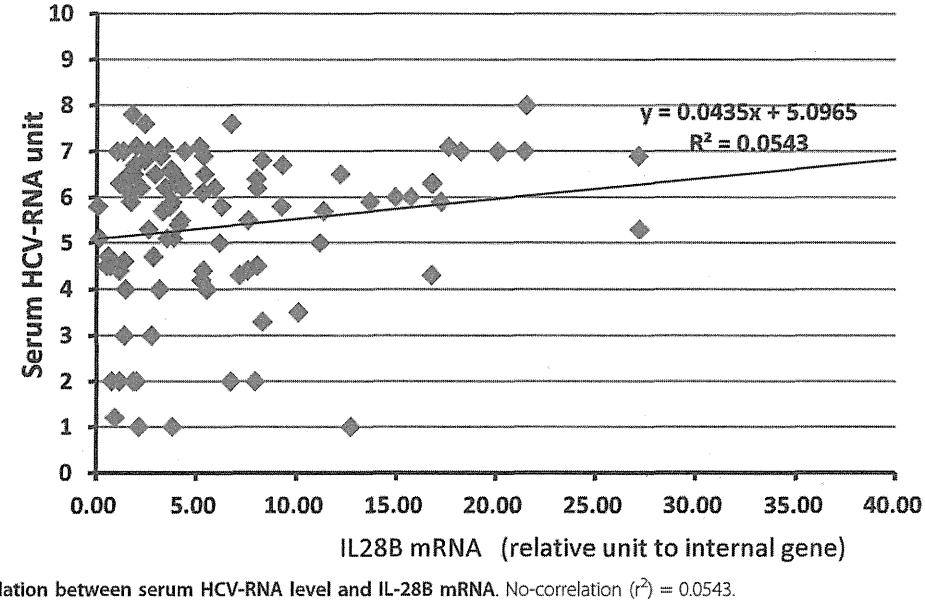

Figure 3 Correlation between serum HCV-RNA level and IL-28B mRNA. No-correlation (r^2) = 0.0543.

Table 2 Assessment by odds ratio analysis for an outcome if a risk factor is present

	factor			
(A) Outcome	**dependent independent**	**Odds ratio**	**95%CI**	**P**
1) HTLV-1 mono-infection	TT vs TG/GG	1.11	0.62-1.99	0.72
2) Co-infection	TT vs TG/GG	0.54	0.04-6.88	1.00
3) High mRNA Expression*	TT vs TG/GG	6.25	1.16-33.75	0.04
4) ATL	TT vs TG/GG	1.50	0.60-3.75	0.39
(B)				
5) Low mRNA Expression	HTLV-1 mono vs DN	0.34	0.06-2.04	0.24
6) Low mRNA Expression	HCV mono vs DN	0.29	0.07-2.23	0.15
7)) Low mRNA Expression	Co-Inf** vs HTLV-1-mono**	9.5	2.06-43.76	0.004
8) ATL	low expression or not	0.8	0.14-4.74	0.81

(A) Upper 4 lines; assessing the risk of 1) HTLV-1 persistent infection, 2) super-imposed HTLV-1 infection with HCV (co-infection), 3) high IL-28B mRNA expression, and 4) ATL development when the genotype is a risk factor (B) Lower 4 lines; assessing the risk factors described in the outcome, the IL-28B mRNA expression level in peripheral blood (5, 6, and 7), and ATL development (8). Consequently, similarly to HCV, HTLV-1 is associated with up-regulation of IL-28B mRNA along with the TT homozygote, and co-infection with HTLV-1 and HCV paradoxically down-regulates the mRNA level

*; IL-28B Expression level, Co-inf = co-infection with HTLV-1 and HCV, mono = mono-infection

findings suggest that the SNP rs8099917 is not associated with susceptibility to HTLV-1 infection or the development of ATL. On the other hand, all of ATL cell- or HTLV-1-infected T-cell- lines examined were exclusively TT homozygous, implying that HTLV-1-infected cells carrying TT homozygotes may immortalize easily in vitro.

Next, we found a strange phenomenon that the IL-28B mRNA expression levels in peripheral blood were lower in samples with HTLV-1/HCV co-infection than in samples with either HTLV-1 or HCV alone, especially significantly for HTLV-1 mono-infection. In particular, samples carrying TT homozygotes were strongly down-regulated, more than the minor TG hetero- and GG-homozygotes. Why are the mRNA expression levels different in mono- and dual-infection? Although it is not known how rs8099917 affects the action of IL-28B, presumably it alters the immune function to viruses. In addition to a common anti-viral IFN-stimulating signal pathway, HTLV-1 may use an alternative anti-viral pathway like HBV [14], because the HTLV-1 provirus is integrated into host genomic DNA and replicates in distinctive life cycle kinetics. Moreover, ATL originates from Treg cells, which play a central role in suppressing immunity [15]. However, this cannot fully explain the impairment in the HTLV-1 carrier's immunity because no ATL cells are present during the carrier period. Thus, we noted IFN-λ(IL-29, IL-28), which was recently discovered as a type III IFNs with anti-virus ability, anti-tumor and immune responses [16-18].

From our results, the IL-28B expression level was higher in HTLV-1 mono-infected individuals including ATL patients. IFN-λis usually up-regulated through activation of the NF-kappaB pathway after viral infection. Actually, the Sendai virus, an influenza A virus, and others have been demonstrated to activate the NF-kappa B pathway, resulting in up-regulated IL-28B expression [19,20]. Accordingly, the highest up-regulation of IFN-λ3 in HTLV-1 mono-infection may be explained by virtue of a viral protein of HTLV-1 having strong NF-kB activating ability. Moreover, it is instructive that IFN-λhas a potent function to expand Treg cells [21], which are mainly infected with HTLV-1, predisposing development of ATL. However, there has not yet been evidence that co-infection with HCV damages Tax action.

Of IL-28B producing cells in the literature, most cells in the blood are described as having a weak or absent expression under the steady state conditions. Li et al. [9] reported that IL-28B mRNA is not always expressed in virally infected cells. Actually, our findings in HTLV-1-infected cases also showed that at least the main producing cells are likely to be cells other than ATL cells because most cell lines from ATL and some blood samples containing ATL cells were expressed faintly. At present, plasmacytoid dendritic cells are indicated to be the most potent producers of IFN-λs [19]. On the other hand, IFN-λ3 reportedly has the functions of proliferating Treg cells which are the origin of ATL cells, suggesting that HTLV-1 is associated with up-regulation via Treg cells infected with HTLV-1.

In conclusion, we found an unusual phenomenon in that the expression of IL-28B mRNA was affected by not only the IL-28B rs8099917 genotype, but also co-infected HTLV-1 with HCV. This will contribute to a better understanding the enigmatic impairment of immunity in the HTLV-1 carrier state, including co-infection with HTLV-1 and HCV.

Abbreviations

HTLV-1: Human T-cell leukemia virus type -1; HCV: Hepatitis C virus; SNP: Single nucleotide polymorphism; IFN: Interferon; PBMC: Peripheral blood