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Anti-interferon-α neutralizing antibody induced telaprevir resistance under the interferon-α plus telaprevir treatment *in vitro*

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ABSTRACT

Although the development of anti-interferon (IFN)-α neutralizing antibodies (NAbs) is likely to be a common clinical problem for patients with various diseases treated with IFN, anti-IFN-α NAb has been exceptionally considered to have no clinical significance in the treatment of chronic hepatitis C with pegylated IFN-α (Peg-IFN-α). However, we recently clarified that the presence of NAb was associated with a non-response to the Peg-IFN plus ribavirin (RBV) therapy. In this study, we used the HCV-replicon system with genotype 1b, and investigated the role of anti-IFN-α NAb in the response to telaprevir (TVR)-containing new antiviral therapy for hepatitis C virus (HCV). Anti-IFN-α NAb-positive sera specifically inhibited the anti-HCV effects of IFN-α, without any effect on the activity of IFN-β *in vitro*. The NAb-positive sera also inhibited the IFN-α-dependent induction of interferon-stimulated genes, MxA and OAS-1, in a dose-dependent manner. Although TVR monotherapy decreased the HCV-RNA *in vitro*, the HCV-RNA was increased again with the development of TVR-resistant mutations. When IFN-α was administrated with TVR, the replication of HCV was continuously suppressed for more than a month. However, in the presence of anti-IFN-α NAb-positive sera, even when IFN-α was combined with TVR, the levels of HCV-RNA exhibited a time-course similar to that with TVR monotherapy, and HCV with TVR-resistant mutations emerged. In conclusion, our findings suggest that the presence of IFN-α NAb decreases the antiviral effects of IFN-α and may be related to the development of TVR-resistant mutated viruses.

1. Introduction

Antiviral therapy for chronic hepatitis C virus (HCV) infection has progressed remarkably during the past few years [1]. Several protease inhibitors (PIs), including telaprevir (TVR) [2–5], boceprevir [6–8], simeprevir [9,10] and faldaprevir [11,12] have been developed, and pegylated interferon-α (Peg-IFN-α) therapy combined with ribavirin (RBV) and TVR has been approved for clinical use in Japan [13,14]. Combination therapy using these three agents (Peg-IFN-α/RBV/TVR) resulted in a sustained virological response (SVR: complete viral eradication) rate of over 60% in genotype 1 patients with a high viral load. However, there are some patients with a poor response to this triple (Peg-IFN-α/RBV/TVR) therapy, and patients with a non-response (NR) to the previous Peg-IFN-α and RBV therapy (Peg-IFN-α/RBV) are often resistant to the new robust therapy. Many host characteristics, such as age, sex, gene polymorphisms in interleukin-28B (IL28B) and viral factors (the HCV genotype and mutations in the HCV RNA sequence) have been found to be associated with the response to the Peg-IFN-α/RBV treatment [15–19].

Anti-IFN neutralizing antibodies (NAbs), which bind to IFN and interfere with its biological activity by inhibiting the interactions between IFN and its receptor, were previously reported to be responsible for host-related recombinant IFN (rIFN) treatment failure [20–22]. Several studies have suggested that, among chronic hepatitis C patients receiving rIFN-α, anti-IFN-α NAb develop more frequently in the NR patients than in the responders [21,22]. However, unlike treatment with rIFN-α, the presence of anti-IFN-α NAb had been considered to have no clinical significance in the Peg-IFN-α treatment. We recently found that the presence of NAb was significantly associated with a NR to Peg-IFN-α/RBV therapy

Abbreviations: HCV, hepatitis C virus; PI, protease inhibitor; TVR, telaprevir; Peg-IFN-α, pegylated interferon-α; RBV, ribavirin; NR, non-response; SVR, sustained virological response; NAb, neutralizing antibody; rIFN, recombinant IFN.

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[23]. Despite the fact that patients with a NR to Peg-IFN-α/RBV therapy often show an unfavorable response to the more recent triple (Peg-IFN-α/RBV/TVR) therapy [5], the role of anti-IFN-α NAb in the response to telaprevir (TVR)-containing new IFN therapy has not been clarified. We therefore investigated the involvement of NAb in the response to the triple therapy.

2. Materials and methods

2.1. Detection of neutralizing antibodies against IFN-α

The sera of the patients who received the IFN-α-based therapy in our department were used in the present study. The patients agreed to participate in the research studies, and provided written informed consent. The anti-IFN NAbs in the patients' sera were detected by an antiviral assay using FL cells and the Sindbis virus, according to the previously reported methods [23–25]. A positive result of was defined as the ability to neutralize the antiviral activity of 10 laboratory units (LU)/mL of a standard IFN preparation. Since Peg-IFN-α2b was used for the triple therapy in the patients, rIFN-α2b (ProSpec, Rehovot, Israel) was used as the standard to determine the positivity of the anti-IFN-α NAb. The measurement of the serum anti-IFN NAb titer and sequences of HCV-RNA were approved by the ethics committees of the appropriate institutional review boards in accordance with the Declaration of Helsinki (Hyogo College of Medicine Approved No. Hi-78, Hi-92, and Hi-112).

2.2. Anti-HCV effects of IFN with replicon cells

The construct of a subgenomic HCV replicon of genotype 1b, pFK-I_{389}neo/NS3-3′/NK5.1 (pFK/Con1 NK5.1) [25,26], was a generous gift from Dr. Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). Natural IFN-β and telaprevir were kindly provided by Toray Industries (Tokyo, Japan) and Mitsubishi Tanabe Pharma Co., Ltd (Osaka, Japan), respectively.

Con1 replicon cells that produce HCV-RNA of genotype 1b were obtained and cultured according to the previously reported methods [27,28]. Con1 replicon cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and 0.25 mg/mL G418 (Roche Applied Science, Mannheim, Germany) under 5% CO_2. In order to investigate the anti-HCV effects of IFN with replicon cells, Con1 replicon cells were plated at a density of 5×10^4 cells/well in 24-well plates (Iwaki glass, Chiba, Japan) and cultured in the presence of rIFN-α2b (final concentration: 1–1000 IU/mL) (ProSpec, Rehovot, Israel), nIFN-β (final concentration:1–1000 IU/mL), TVR (final concentration: 3 μg/mL), patient serum (final concentration: 0.1–3%) or anti-IFN-α polyclonal antibodies (pAb) (PBL Biomedical Laboratories, Piscataway, NJ), or anti-IFN-β pAb (CHEMICON International, Temecula, CA). The sera of the patients who received the IFN-α based therapy in our department were used in the present study. The patients agreed to participate in the research studies, including the genomic research, and provided written informed consent.

2.3. Quantification of HCV RNA or mRNAs of interferon-stimulating genes by real-time RT-PCR

Total RNA was isolated using the RNeasy Plus mini kit (QIAGEN, Valencia, CA), and cDNA was generated with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. HVC-RNA and interferon-stimulating gene (ISG) mRNAs were quantified using the ABI 7500 FAST system with the following Taqman gene expression assay regents (Applied Biosystems) according to the manufacturer's instructions: GAPDH-VIC (Hs02758991_g1), HCV 5′UTR-FAM (Pa03453408_s1), MxA-FAM (Hs00895608_m1) and OAS1-FAM (Hs00973637_m1). The results were normalized to the GAPDH expression and were expressed relative to control cells.

2.4. Amplification and sequencing of the gene encoding the NS3 protease catalytic domain from Con1

The region encoding the NS3 protease catalytic domain was amplified by nested reverse-transcription polymerase chain reaction (PCR) from the Con1-replicon cells. After isolation of the total RNA from Con1-replicon cells using RNeasy Plus Mini Kit (QIAGEN), the fragments of the target sequence were amplified using a commercial one-step reverse transcription PCR kit (Superscript III RNase H-Reverse Transcriptase with High Fidelity Platinum Taq DNA Polymerase; Invitrogen) and the following primers: Con1-NS3-1 (5′-ATTACGGCCTACTCCCAACAG-3′) and Con1-NS3-3 (5′-CACCTGGAATGTCTGCGGTA-3′).

The RT-PCR product was subjected to second-round PCR using Platinum PCR Super Mix (Invitrogen) and an inner set of the following primers: Con1-NS3-2(5′-TGCATCATCACTAGCCTCACA-3′) and Con1 NS3-4 (5′–GGACGAGTTGTCCGTGAAGA-3′). The amplified fragments were visualized by agarose gel electrophoresis and ethidium bromide staining. The DNA from the nested-PCR then was separated on a 1% agarose gel, and the appropriately sized product was purified with a MinElute PCR Purification Kit (QIAGEN). The sequences of the amplified fragments were determined by direct sequence without subcloning using a BigDye® Terminator v3.1 Cycle Sequencing Kit and an ABI Genetic Analyzer 3130xl (Applied Biosystems, Inc, Japan). Our experimental study was approved by the institutional review board (Hyogo College of Medicine Approved No. 211001).

3. Results

3.1. The sera of patients with IFN-α-NAb specifically inhibited the biological activity of IFN-α

We previously reported the association of IFN-α NAb with a NR to Peg-IFN-α/RBV therapy. In the present study, we investigated the role of IFN-α NAb in the response to TVR-containing therapy *in vitro*. We used the HCV-replicon system with genotype 1b, and confirmed that both IFN-α and IFN-β inhibited the replication of HCV *in vitro* in a dose-dependent manner (Fig. 1A). The sera of patients with IFN-α NAb reduced the antiviral activity of IFN-α in a dose-dependent manner, while the anti-HCV activity of IFN-β was not affected by the NAb-positive sera (Fig. 1B).

We further investigated the effects of IFN-α NAb-positive sera on the expression of the ISGs. The NAb-positive sera decreased the IFN-α-dependent induction of ISGs in a dose-dependent manner (Fig. 2), thus indicating that the IFN-α NAb-positive sera inhibited the intra-cellular signaling of IFN-α in HCV replicon-transfected hepatic cells.

3.2. Anti-INF-α NAb inhibits IFN-α from preventing acquired resistance to TVR

In order to investigate the role of anti-IFN-α NAb in the response to TVR-containing antiviral treatment, HCV replicon-transfected cells were treated with TVR alone, IFN-α alone or their combination under the conditions which were consistent with the blood concentrations observed in clinical practice (IFN-α: 60 IU/mL; TVR: 3 μg/mL), and then the HCV-RNA levels were evaluated. The exposure to TVR alone markedly decreased the HCV-RNA levels after the initiation of the treatment (Fig. 3A, days 7 and

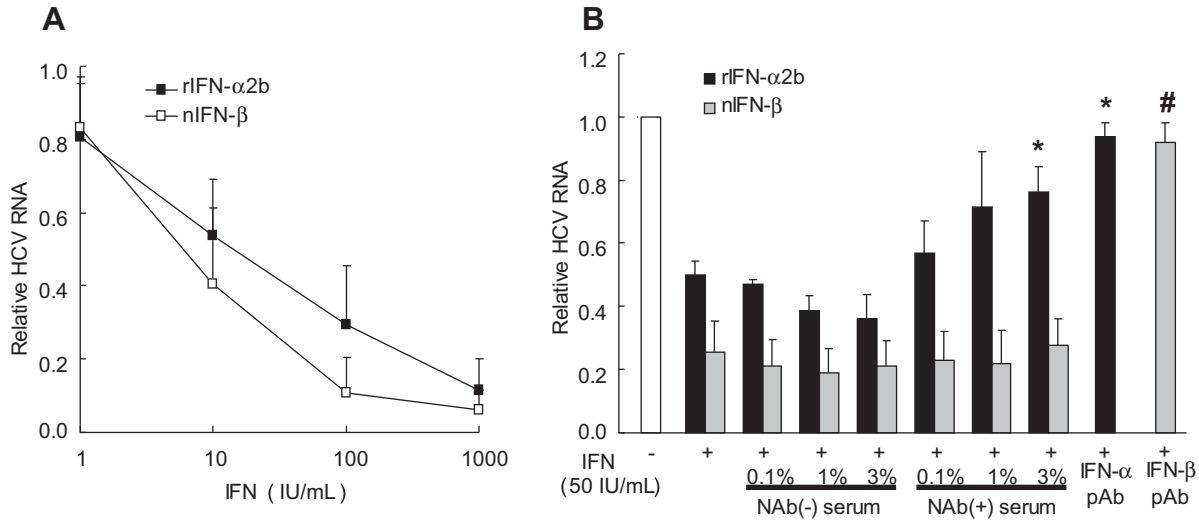

Fig. 1. The effects of IFN-α-neutralizing antibodies on the anti-HCV effects of interferon-α and interferon-β. (A) HCV-replicon cells were treated with either IFN-α or IFN-β for 48 h. Both types of IFNs suppressed HCV-RNA replication in a dose-dependent manner. (B) HCV-replicon cells were treated with either IFN-α or IFN-β for 48 h. Irrespective of IFN-α treatment, commercially available polyclonal antibodies (pAb) against IFN-α inhibited the anti-HCV effect of IFN-α and led to increased HCV replication (pAb: black bar). A commercially available pAb for IFN-β also inhibited the anti-HCV effects of IFN-β (pAb: gray bar). The IFN-α-NAb-negative sera did not affect the anti-HCV effects of IFN-α or IFN-β; however, IFN-α NAb-positive sera specifically inhibited the anti-HCV effects of IFN-α without any effect on the anti-HCV effects of IFN-β. $^{*}P < 0.05$ control (IFN-α treatment alone) versus treatment with IFN-α NAb-positive sera or pAbs, as determined by a non-repeated measurements ANOVA with a subsequent Bonferroni correction. # The IFN-β pAb-treated group only showed significantly lower HCV-RNA levels when compared with the control (IFN-β treatment alone) group ($P < 0.05$) by a non-repeated measures ANOVA with a subsequent Bonferroni correction.

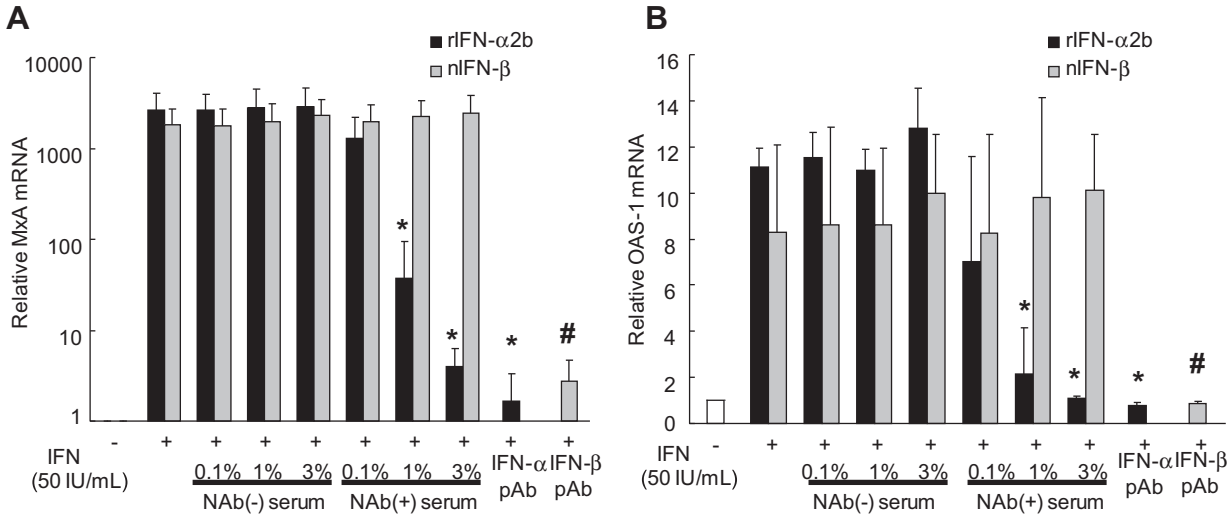

Fig. 2. The effects of the IFN-α-neutralizing antibodies on the expression of interferon-stimulating genes (ISGs) induced by interferon-α and interferon-β. The expression levels of two interferon-stimulating genes (ISGs) were evaluated with a real-time PCR method, as described in Section 2. (A) HCV-replicon cells were cultured under various conditions for 48 h. Irrespective of IFN-α treatment, commercially available polyclonal antibodies (pAb) against IFN-α suppressed the induction of the IFN-α-inducible gene, MxA (pAb: black bar). A commercially available pAb for IFN-β also suppressed the induction of the MxA gene by IFN-β (pAb: gray bar). IFN-α NAb-negative sera did not affect the induction of the MxA gene by IFN-α or IFN-β, whereas IFN-α-NAb-positive sera specifically inhibited the IFN-α-dependent induction of MxA, without any influence on the gene induction by IFN-β. $^{*}P < 0.05$ control (IFN-α treatment alone) versus cells treated with IFN-α NAb-positive sera or pAb, as determined by a non-repeated measures ANOVA with a subsequent Bonferroni correction. # The IFN-β pAb-treated group only showed significantly lower expression of MxA gene when compared with the control (IFN-β treatment alone) group ($P < 0.05$) by a non-repeated measures ANOVA with a subsequent Bonferroni correction. (B) IFN-α NAb-negative sera did not affect the induction of the OAS-1 gene by IFN-α and IFN-β, whereas IFN-α NAb-positive sera specifically inhibited the IFN-α-dependent induction of the OAS-1 gene, without any influence on the gene induction by IFN-β. $^{*}P < 0.05$ control cells (IFN-α treatment alone) versus those treated with IFN-α NAb-positive sera or pAb, as determined by a non-repeated measures ANOVA with a subsequent Bonferroni correction. # The IFN-β pAb-treated group only showed significantly lower expression of OAS-1 gene when compared with the control (IFN-β treatment alone) group ($P < 0.05$) by a non-repeated measures ANOVA with a subsequent Bonferroni correction.

14), but then the HCV-RNA levels returned to the pretreatment levels (Fig. 3A, days 21 to 35), suggesting that TVR alone lost efficacy during treatment because of acquired resistance to TVR. Treatment with IFN-α alone mildly decreased the HCV-RNA levels (less than a one log drop) (Fig. 3A), suggesting that IFN-α alone is not sufficient to suppress the replication of HCV with genotype 1 *in vitro*. However, combination treatment with TVR and IFN-α resulted in continuous suppression of the HCV levels for more than 1 month (Fig. 3A). These results suggest that TVR alone resulted in viral breakthrough, while additional IFN-α maintained the anti-HCV effects of TVR by preventing the emergence of TVR-resistant viruses.

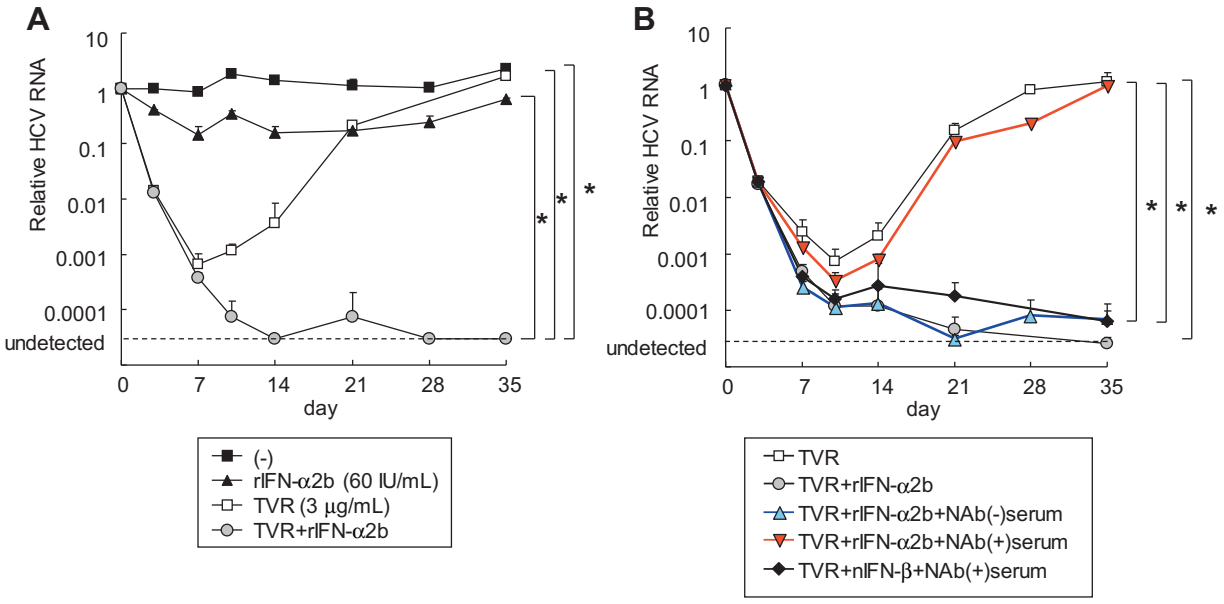

Fig. 3. The sera of patients with IFN-α-NAb specifically inhibited the anti-HCV activity of IFN-α. HCV replicon-transfected cells were treated with TVR alone, IFN-α alone or their combination, and the HCV-RNA levels were measured. In order to evaluate the time course of the HCV-RNA levels, continuously-cultivated cells were harvested at the indicated times for the isolation of total RNA. (A) The administration of TVR alone remarkably decreased the HCV-RNA levels after the initiation of the treatment (days 7 and 14), but then the HCV-RNA levels returned to the pretreatment levels (days from 21 to 35), suggesting that TVR monotherapy lost its antiviral effects because of acquired resistance to TVR. Although treatment with low-dose IFN-α alone barely decreased the HCV-RNA levels, combination treatment with TVR and IFN-α resulted in continuous suppression of the HCV levels for more than 1 month. Therefore, TVR alone resulted in viral breakthrough, while additional IFN-α maintained the anti-HCV effects of TVR by preventing the emergence of TVR-resistant viruses. The HCV-RNA level of the TVR + rIFN-a2b-treated cells was significantly lower than that of the control (no treatment), rIFN-a2b alone and TVR alone cells (*$P < 0.05$ by a non-repeated measures ANOVA with a subsequent Bonferroni correction). (B) HCV replicon-transfected cells were treated with anti-IFN-α NAb-positive or -negative sera concomitantly with IFN-α/TVR. Although the HCV-RNA levels markedly decreased, irrespective of the positivity of anti-IFN-α NAb in the sera, the HCV-RNA level was re-increased only when anti-IFN-α NAb-positive sera were administered. The time course curve of the HCV-RNA levels in the presence of anti-IFN-α NAb-positive sera (plus IFN-α and TVR) was indistinguishable from that following treatment with TVR alone. However, anti-IFN-α NAb-positive sera did not affect the anti-HCV effects of IFN-β plus TVR treatment. Therefore, anti-IFN-α NAb specifically abolished the preventive effect of IFN-α on the viral breakthrough. The HCV-RNA level was significantly higher in cells treated with TVR + rIFN-α2b + NAb-positive serum than in cells treated with TVR + rIFN-α2b, with TVR + rIFN-α2b + NAb-negative serum, or with TVR + nIFN-β + NAb-positive serum on day 35 (*$P < 0.05$ by a non-repeated measures ANOVA with a subsequent Bonferroni correction.).

We further examined whether sera from anti-IFN-α NAb-positive patients diminished the sustained suppression of HCV-RNA by the combination treatment with IFN-α and TVR. HCV replicon-transfected cells were treated with anti-IFN-α NAb-positive or -negative sera concomitantly with IFN-α/TVR. Although the HCV-RNA levels remarkably decreased, irrespective of the positivity of the anti-IFN-α NAb in the sera, the HCV-RNA level increased again only when the anti-IFN-α NAb-positive sera were administered (Fig. 3B). The longitudinal changes in the HCV-RNA levels in the presence of anti-IFN-α NAb-positive sera (plus IFN-α and TVR) were indistinguishable from those following treatment with TVR alone. However, anti-IFN-α NAb-positive sera did not affect the antiviral effects of IFN-β plus TVR treatment (Fig. 3B). Taken together, these results suggest that the anti-IFN-α NAb specifically inhibits the preventive effects of IFN-α on the emergence of TVR-resistant HCV.

3.3. Inactivation of IFN-α by the NAb causes resistance-related mutations in the NS3 protease gene of HCV

Mutations in the NS3 protease gene of HCV are responsible for acquired resistance to protease inhibitors, such as TVR, which target the NS3 protein product [29–32]. We therefore performed direct sequencing to examine whether the resistance to IFN-α/TVR caused by the anti-IFN-α NAb was related to the development of mutations in the NS3 gene. In agreement with the previous reports [29–32], in HCV replicon-transfected cells treated with TVR alone *in vitro*, the TVR-resistant HCV had mutations at the nucleic acid sequence GCT corresponding to the 156th alanine of the NS3, which was substituted with GTT (A156V), ACT (A156T) or TCT (A156S) (Fig. 4; upper panel).

Next, HCV replicon-transfected cells were treated with IFN-α/TVR in the presence of anti-IFN-α NAb-positive or -negative sera. The anti-IFN-α NAb-negative sera did not inhibit the antiviral effects of IFN-α/TVR (as shown in Fig. 3), and no mutations were

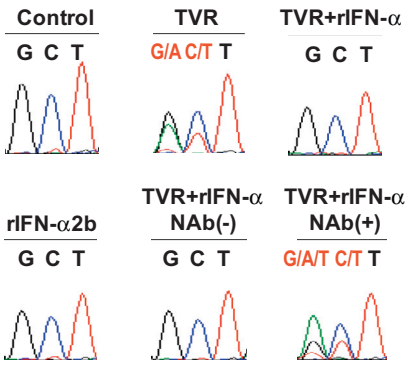

Fig. 4. Inactivation of IFN-α with the IFN-α NAb induced TVR-resistant mutations in the NS3 protease gene of HCV. Since viral breakthrough was observed following the treatment with TVR alone, we investigated the sequences of HCV-RNA (on day 35 in Fig. 4). The treatment with TVR alone induced HCV with TVR-resistant mutations (GTC → GTT: A156V and GTC → ACT: A156T) (upper panel). Irrespective of the presence of IFN-α, TVR-resistant mutations (GTC → GTT: A156V, GTC → ACT: A156T, and GTC → TCT: A156S) appeared in the presence of NAb-positive sera (lower panel). These findings suggested that the inactivation of IFN-α by the NAb would cause a condition similar to the TVR monotherapy, resulting in the emergence of TVR-resistant viruses.

observed in the NS3 gene (Fig. 4; lower panel). However, treatment with anti-IFN-α NAb-positive sera was associated not only with TVR resistance, but also with mutations of the GCT sequence in the NS3 gene into GTT (A156V), ACT (A156T) or TCT (A156S) (Fig. 4; lower panel).

4. Discussion

Although the development of anti-IFN NAbs has been considered to be a common clinical problem in patients with several diseases treated with IFN, including leukemia, multiple sclerosis and chronic hepatitis C [23,25], anti-IFN-α NAb had been exceptionally considered to have no clinical significance in the treatment with Peg-IFN-α. However, we recently found that anti-IFN-α NAb were associated with a NR to Peg-IFN-α/RBV treatment in patients with chronic hepatitis C [23]. Our present *in vitro* studies demonstrated that, in the presence of anti-IFN-αNAb, the antiviral effects of IFN-α were decreased, and TVR-resistant mutations were induced (Figs. 3 and 4). Since TVR monotherapy was shown to frequently cause viral breakthrough and lead to treatment failure in patients with hepatitis C virus genotype 1b infections [33], our findings suggest that the inhibition of the biological activity of IFN-α by NAb could be involved in the undesirable treatment outcome of the new triple therapy.

It has been reported that the anti-HCV effects of Peg-IFN-α/RBV/TVR are higher than those of Peg-IFN-α/TVR (without RBV) [2]. However, the mechanism(s) by which RBV amplifies the anti-HCV effects of IFN have not been fully clarified, and it is difficult to evaluate how the supportive effect of RBV on the anti-HCV activity of IFN would be affected by NAb *in vitro*. Therefore, the present *in vitro* study did not use RBV, and focused on analyzing the antiviral effects of TVR when the antiviral effects of IFN-α were inhibited by NAb. Although we showed the development of TVR-resistant viruses in the presence of the NAb, our results were obtained without RBV treatment, and did not directly reflect the *in vivo* conditions. In addition, we do not have a system to perform deep sequencing, which would provide a better understanding of the dynamics of the HCV mutations [34–36], and therefore, our analysis regarding TVR-resistant mutations depends on the conventional direct sequence method. Although our findings would provide an important suggestion for clinical setting, the role of NAb in the triple therapy will need to be demonstrated by a clinical study with a large number of patients and the introduction of more advanced analytical methods.

In summary, our *in vitro* studies suggest that the presence of IFN-α NAb should decrease the antiviral effects of IFN-α and result in the emergence of TVR-resistant mutated viruses. This is the first report that shows the possible involvement of anti-IFN-αNAb in the new antiviral therapy.

Conflict of interest statement

Shuhei Nishiguchi received financial support from Chugai Pharmaceutical, MSD, Dainippon Sumitomo Pharma, Ajinomoto Pharmaceuticals and Otsuka Pharmaceutical. Hiroko Iijima received financial support from Chugai Pharmaceutical. Chisa Kuga is an employee of Toray Industries.

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