Anticancer Chemotherapy Inhibits MHC Class I–Related Chain A Ectodomain Shedding by Downregulating ADAM10 Expression in Hepatocellular Carcinoma

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Abstract

MHC class I–related chain A (MICA) is a ligand for the NKG2D-activating immunoreceptor that mediates activation of natural killer (NK) cells. The ectodomain of MICA is shed from tumor cells, which may be an important means of evading antitumor immunity. We previously reported that patients with hepatocellular carcinoma (HCC) display high levels of soluble MICA in circulation, which could be downregulated by chemotherapy. The present study shows that anti-HCC drugs suppress MICA ectodomain shedding by inhibiting expression of a disintegrin and metalloproteinase 10 (ADAM10). Both ADAM10 and CD44, a typical substrate of the ADAM10 protease, were expressed in human HCC tissues and HCC cells but not in normal liver tissues or cultured hepatocytes. Small interfering RNA–mediated knockdown experiments revealed that ADAM10 is a critical sheddase for both MICA and CD44 in HCC cells. Of interest is the finding that epirubicin clearly downregulated ADAM10 expression and MICA shedding in HCC cells; its suppressive effect on MICA shedding was abolished in ADAM10-depleted cells. Epirubicin treatment also enhanced the NKG2D-mediated NK sensitivity of HCC cells. Patients with HCC had significantly higher levels of serum-soluble CD44, which correlated well with serum-soluble MICA levels, thus suggesting a close link between ADAM10 activity and MICA shedding in these patients. Soluble MICA and CD44 levels were downregulated with a significant correlation in patients treated by transarterial chemoembolization using epirubicin. In conclusion, anticancer drugs can modulate expression of ADAM10, which is critically involved in MICA ectodomain shedding. Epirubicin therapy may have a previously unrecognized effect on antitumor immunity in HCC patients. [Cancer Res 2009;69(20):8050–7]

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and nonalcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis, in particular, being considered a premalignant condition (1, 2). With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas transcatheter arterial chemoembolization (TACE) is a well-established technique for more advanced HCC (3). The liver contains a large compartment of innate immune cells [natural killer (NK) cells and natural killer T cells] and acquired immune cells (T cells; refs. 4, 5), but the activation of these immune cells after HCC treatments remains unclear. If such treatments can efficiently activate abundant immune cells in the liver, this could lead to the establishment of attractive new strategies for HCC treatment.

MHC class I–related chain A and B (MICA and MICB) are ligands for NKG2D expressed on a variety of immune cells (6). In contrast to classic MHC class I molecules, MICA/B are rarely expressed on normal cells but frequently on tumor cells (7–10). The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic activity and cytokine production (11). Thus, the MICA/B-NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells (12). In addition to those membrane-bound forms, MICA/B molecules are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy (13–15). Soluble MICA/B in circulation downregulates NKG2D expression and disturbs NKG2D-mediated antitumor immunity (9, 10, 13). We previously reported that soluble MICA could be detected in sera of HCC patients (16) and that TACE treatment reduces the levels of soluble MICA and thereby upregulates the expression of NKG2D (17). Thus, cancer therapy may have a beneficial effect on NKG2D-mediated immune responses.

The release of soluble MICA/B from tumor cells is impaired by metalloproteinase inhibitors, suggesting the involvement of members of the metzincin superfamily, such as ADAM proteins (14, 18). In addition, ERp5, related to protein disulfide isomerase, is required for the MICA shedding as it reduces disulfide bond of the α3 domain of MICA (19). Although it may not be a direct protease for MICA, it may enable proteolytic cleavage through conformational change. Recently, it was reported that MICA shedding of 293T fibroblast cells and HeLa cervical cancer cells was inhibited by silencing of the ADAM10 and ADAM17 proteases (20). This suggests that ADAM family proteins may be a therapeutic target for enhancing antitumor immunity, but how to therapeutically modulate these proteins is still not clear. Furthermore, it remains to be determined whether ADAMs can regulate MICA shedding in a clinical setting.

In the present study, we showed that ADAM10, but not ADAM17, was critically required for MICA shedding in human HCC cells. Of importance is the discovery that epirubicin, a widely used anti-HCC drug, was capable of downregulating ADAM10 expression and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-0789

activity in HCC cells; it can thus inhibit MICA shedding and enhance NK sensitivity. ADAM10 was immunohistochemically detected in HCC tissues and a correlation was observed between soluble MICA levels and ADAM10 activity determined by soluble CD44 levels in HCC patients. The present study sheds light on previously unrecognized effects of an anticancer drug on modulating ADAM family proteins and MICA shedding and thus suggests a promising aspect for chemoimmunotherapy against human HCC.

Materials and Methods

Liver tissues and immunohistochemistry. Human HCC tissues (n = 8) and normal liver tissues (n = 2) obtained at surgical resection were used. Informed consent, under an institutional review board–approved protocol,

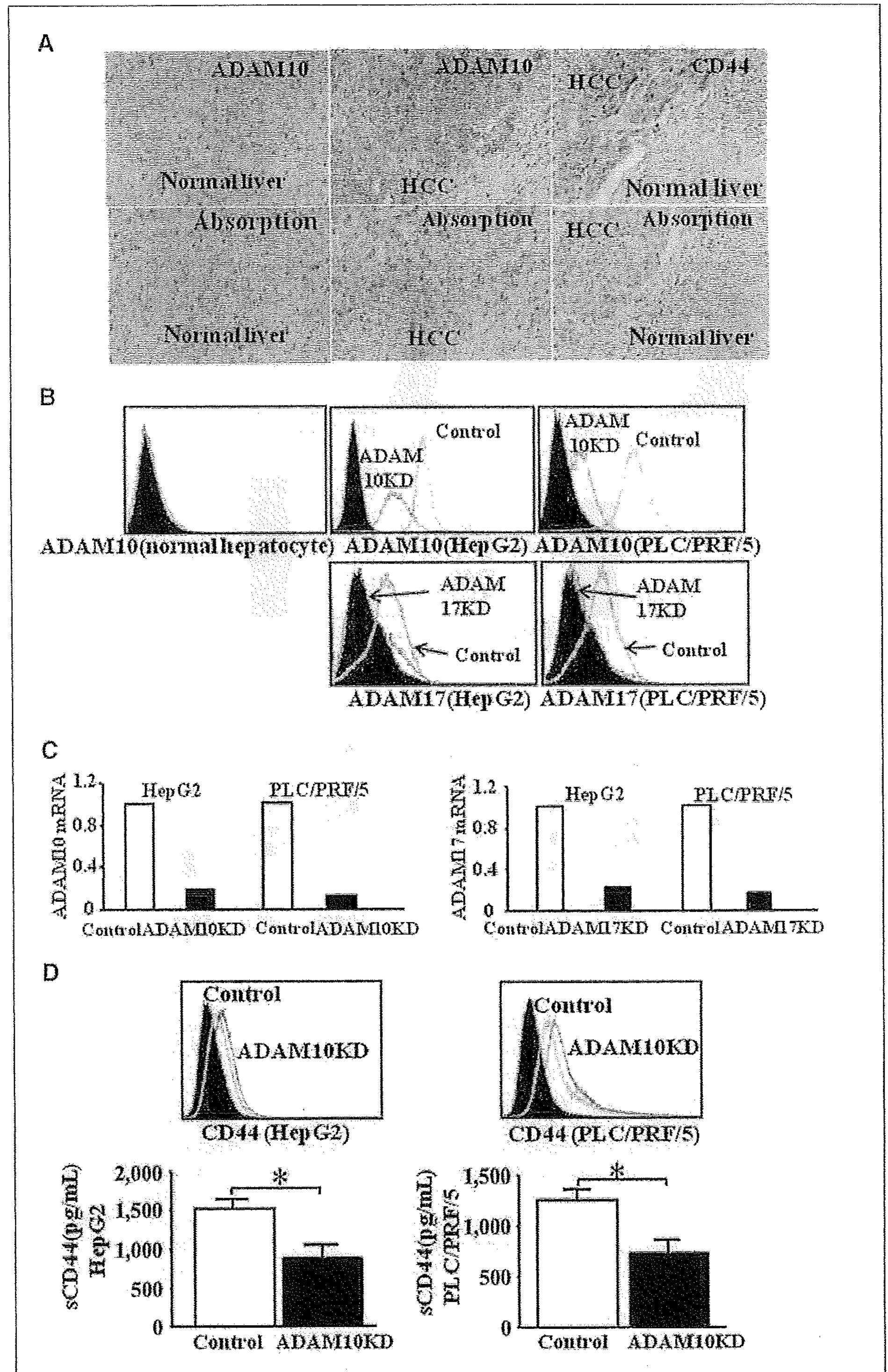

Figure 1. Expression of ADAM10 and CD44 in human HCC tissues and ADAM10 or ADAM17 knockdown in human HCC cells. *A*, immunohistochemical detection of ADAM10 and CD44 in human HCC tissues (n = 8) and normal liver tissues (n = 2). Liver sections were stained with the corresponding antibodies (*top panels*). Both primary antibodies were incubated with recombinant CD44 and ADAM10 proteins and then applied to liver sections in parallel as the absorption test (*bottom panels*). Representative images are shown. *B* and *C*, expression of ADAM10 or ADAM17 in human primary hepatocyte and HCC cell lines (*HepG2* and *PLC/PRF/5*). Cells were treated with ADAM10 siRNA, ADAM17 siRNA, or control siRNA, and subjected to analysis of ADAM10 or ADAM17 expression by flow cytometry (*B*) or real-time RT-PCR (*C*). *Histograms*, anti-ADAM10 or anti-ADAM17 staining of ADAM10 or ADAM17 siRNA-treated cells (*ADAM10KD* or *ADAM17KD, black dotted line*) and control siRNA-treated cells (*Control, gray line*), respectively. *Closed histograms*, control IgG staining. *D*, the expression of membrane-bound CD44 on HCC cells treated with ADAM10 siRNA (ADAM10KD, *black line*) or control siRNA (*Control, gray line*) was evaluated by flow cytometry (*top panels*). *Closed histograms*, control IgG staining. Soluble CD44 (*sCD44*) production from HCC cells treated with ADAM10 siRNA or control siRNA were evaluated by specific ELISA (*bottom panels*). *, $P < 0.05$.

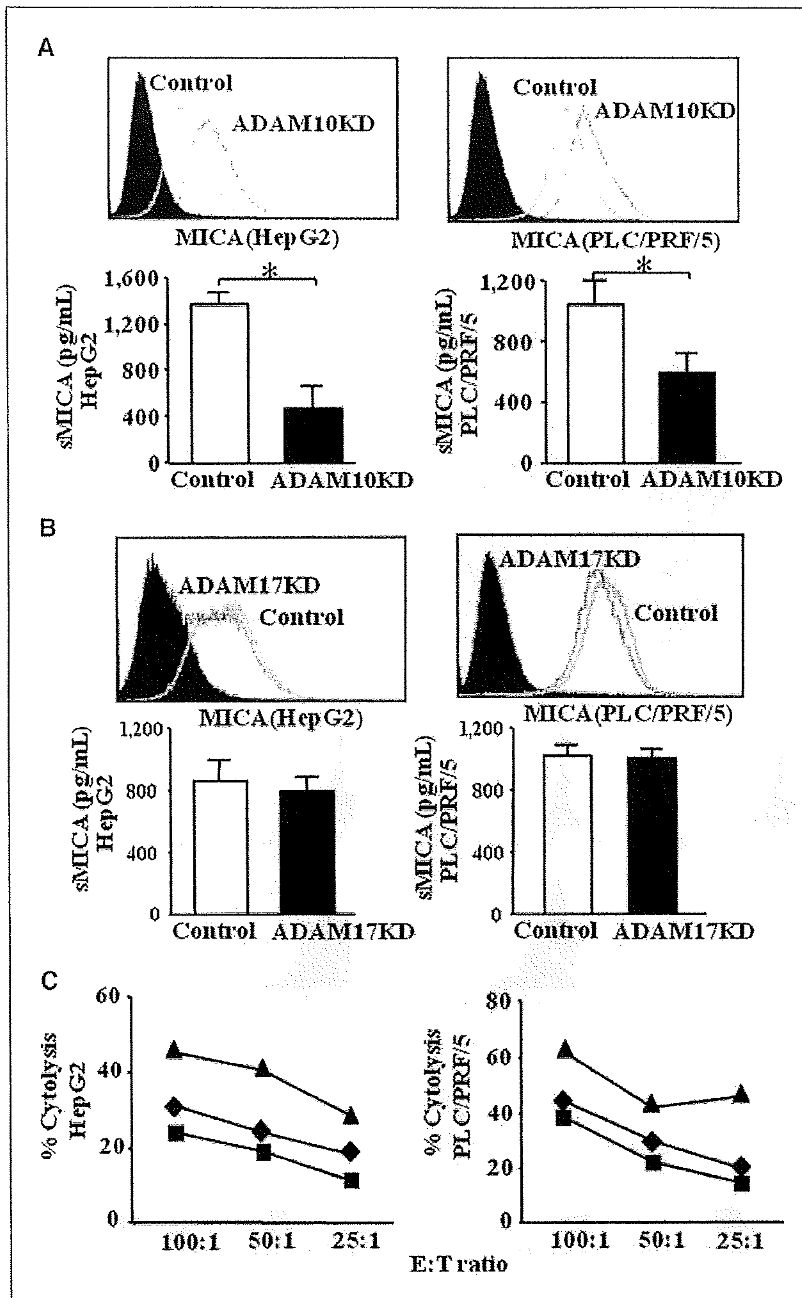

Figure 2. Expression of MICA in ADAM10 or ADAM17 knockdown HCC cells and NK sensitivity in ADAM10 knockdown HCC cells. *A* and *B*, the expression of membrane-bound MICA on HCC cells treated with ADAM10 siRNA (*ADAM10KD, black line*; *A*), ADAM17 siRNA (*ADAM17KD, black line*; *B*), or control siRNA (*Control, gray line*) was evaluated by flow cytometry (*top panels*). *Closed histograms,* control IgG staining. Soluble MICA (*sMICA*) production from HCC cells treated with ADAM10 siRNA (*A*), ADAM17 siRNA (*B*), or control siRNA were evaluated by specific ELISA (*bottom panels*). *, $P < 0.05$. *C*, HCC cells treated with ADAM10 siRNA or control siRNA were subjected to ^{51}Cr-release assay against NK cells. Cytolytic activity of NK cells against control HCC cells (■) or ADAM10 knockdown HCC cells without (▲) or with blocking antibody of MICA/B (6D4; ♦). Representative results are shown. Similar results were obtained from three independent experiments.

was obtained from all patients before sample acquisition. Liver sections were subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA). The primary antibodies used were anti-ADAM10 and anti-CD44 (R&D Systems). To confirm the specificity of the staining, primary antibodies were incubated with recombinant CD44 or ADAM10 protein (R&D Systems, Minneapolis, MN) for 3 h and then applied onto liver sections in parallel with staining of the primary antibodies as the absorption test.

HCC cell lines. Human HCC cell lines HepG2 and PLC/PRF/5 were purchased from the American Type Culture Collection and were cultured with DMEM supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY) in a humidified incubator at 5% CO_2 and 37°C.

RNA silencing. The small interfering RNA (siRNA) method was used to knockdown ADAM10 and ADAM17. Stealth RNAi oligonucleotide targeting ADAM10 or ADAM17 and scrambled oligonucleotides as a control were purchased from Invitrogen (Carlsbad, CA). Cells were transfected by RNAi Max transfection reagent (Invitrogen) with 50 nmol/L siRNA. At 24 h posttransfection, the cells were analyzed for specific depletion of the mRNAs of ADAM10 and ADAM17 by real-time reverse transcription-PCR (RT-PCR; Applied Biosystems, Foster City, CA). The following siRNAs were used: ADAM10, 5′-AUAUCUGGGCAAUCACAG-CUUCUCG-3′; scramble control, 5′-AUACUUGGUCAACGCACUUCGAUGG-3′; ADAM17, 5′-UGAACAAGCUCUUCAGGUGGUUCUC-3′; scramble control, 5′-UGAUUAGAACUCUCGACUGGUGCUC-3′.

ELISA. The supernatants of cultured cells were harvested at 24 h after transfection with siRNA as well as sera from HCC patients (n = 97) and age-matched healthy volunteers (n = 32) were subjected to analysis of soluble MICA and soluble CD44 levels. Informed consent, under an institutional review board–approved protocol, was obtained from all patients before sample acquisition. The levels of soluble MICA and soluble CD44 were

determined by DuoSet MICA eELISA kit (R&D Systems) and soluble CD44std ELISA (Abcam, Cambridge, MA), respectively.

Flow cytometry. For the detection of membrane-bound MICA and CD44, cells were incubated with an anti-MICA–specific antibody (2C10, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CD44 antibody (R&D Systems) and stained with phycoerythrin (PE)-goat anti-mouse immunoglobulin (Beckman Coulter) as a secondary reagent and then subjected to flow cytometric analysis. For the detection of ADAM10 or ADAM17, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) and stained with PE-conjugated anti-ADAM10 or anti-ADAM17 antibody (R&D Systems). Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson).

Plasmid construction of pMyc-MICA. MICA full coding cDNA was isolated from Huh7, human HCC cells, using a conventional RT-PCR method (Supplementary Fig. S1, DDBJ/EMBL/Genbank accession number AB506764) and inserted into the *Hin*dIII-*Xba*I site of pcDNA3 (Invitrogen). A C-myc tag was placed between the leader peptide and the α1 domain of MICA by site-specific mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) referred to as pMyc-MICA. Cells were transfected with pMyc-MICA using a Lipofectamine LTX reagent (Invitrogen). The green fluorescent protein (GFP)–expressing vector (pEGFP-C1, Clontech, Mountain View, CA) was cotransfected to evaluate the transfection efficiency.

Immunoprecipitation. Cells or tissues were homogenized in lysis buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/mL aprotinin, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and PBS. To the cell supernatants, 0.5% NP40 and a cocktail of protease inhibitors were added. The protein contents of the samples were determined by BCA protein assay kit (Pierce, Rackford, IL). Immunoprecipitation with anti–c-Myc beads was performed for 1 h at 4°C. Immunocomplexes were eluted by a c-Myc–tagged peptide solution (MBL, Woburn, MA). The samples after immunoprecipitation were treated with 250 mU of N-glycosidase F (Roche, Mannheim, Germany) for 3 h at 37°C.

Western blotting. The total cellular protein was electrophoretically separated using SDS-12% polyacrylamide gels and transferred onto polyvinylidene difluoride membrane. The membrane was blocked in TBS-Tween containing 5% skim milk for 1 h and then probed with anti-Myc mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA) at 4°C overnight. Horseradish peroxidase–conjugated anti-rabbit antibody and SuperSignal West Pico System (Pierce) were used for the detection of blots.

Real-time RT-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen). The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Ready-to-use assays (Applied Biosystems) were used for the quantification of ADAM10 (Hs00153853_m1), ADAM17 (Hs00234221_m1), MICA (Hs00792195_m1), β-actin (Hs99999903_m1), and CD44 (Hs00174139_m1) mRNAs according to the manufacturer's instructions. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. β-Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

WST-8 assay. HepG2 and PLC/PRF/5 cells were treated with different concentrations of epirubicin for 24 h. Cell growth of epirubicin-treated HCC cells was determined by WST-8 assay (Nacalai Tesque, Kyoto, Japan) as previously described (21).

NK cell analysis. NK cells were isolated from human peripheral blood mononuclear cells by magnetic cell sorting using CD56 MicroBeads (Miltenyl Biotech, Auburn, CA) as previously described (16). The cytolytic ability of NK cells was assessed by 4-h ^{51}Cr-releasing assay with or without MICA/B-blocking antibody (6D4; ref. 7), which binds to the α1 and α2 domains of MICA and MICB. 6D4 was a generous gift from Drs. Veronika Groh and Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA).

Statistics. All values were expressed as the mean and SD. The statistical significance of differences between the groups was determined by applying Student's *t* test or two-sample *t* test with Welch correction after each group

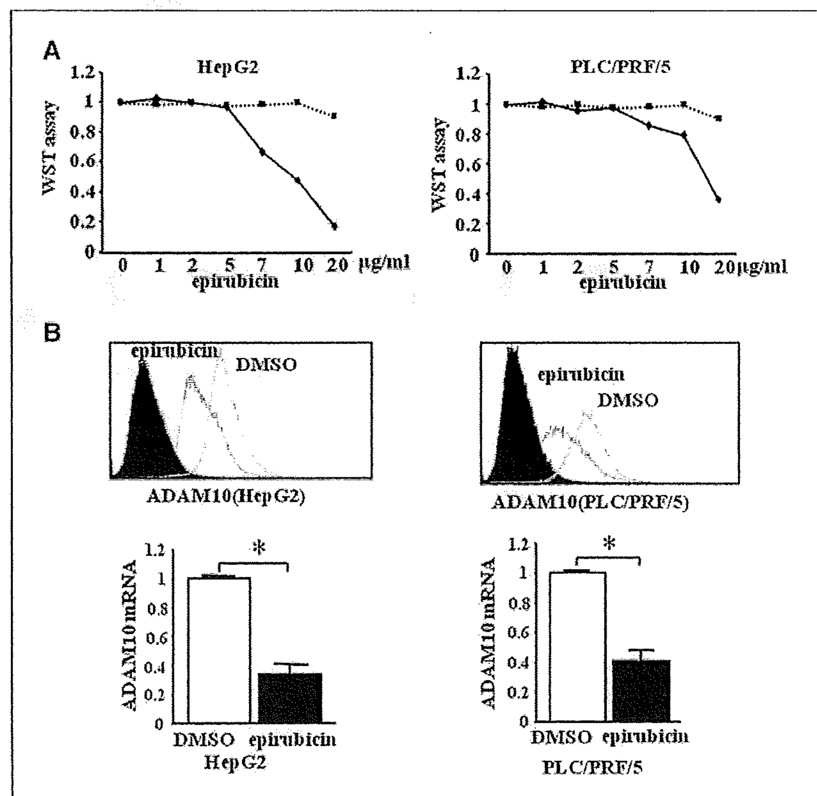

Figure 3. Expression of ADAM10 in epirubicin-treated HCC cells. *A,* the cytotoxicity of epirubicin to human HCC cells was evaluated by WST-8 assay. Cells were treated with different doses of epirubicin (*solid lines*) or vehicle (*DMSO; dotted lines*) for 24 h, and the viability of the cells was evaluated by the WST-8 assay. *B,* ADAM10 expression of epirubicin-treated HCC cells. Cells were treated with a nontoxic dose of 1 μg/mL epirubicin (*black lines*) or vehicle (DMSO, *gray lines*) for 24 h and their ADAM10 expression was evaluated by flow cytometry (*top panels*). *Closed histograms,* control IgG staining. Total RNA was extracted at 24 h of epirubicin treatment and mRNA levels of ADAM10 were evaluated by real-time RT-PCR (*bottom panels*). *, $P < 0.05$.

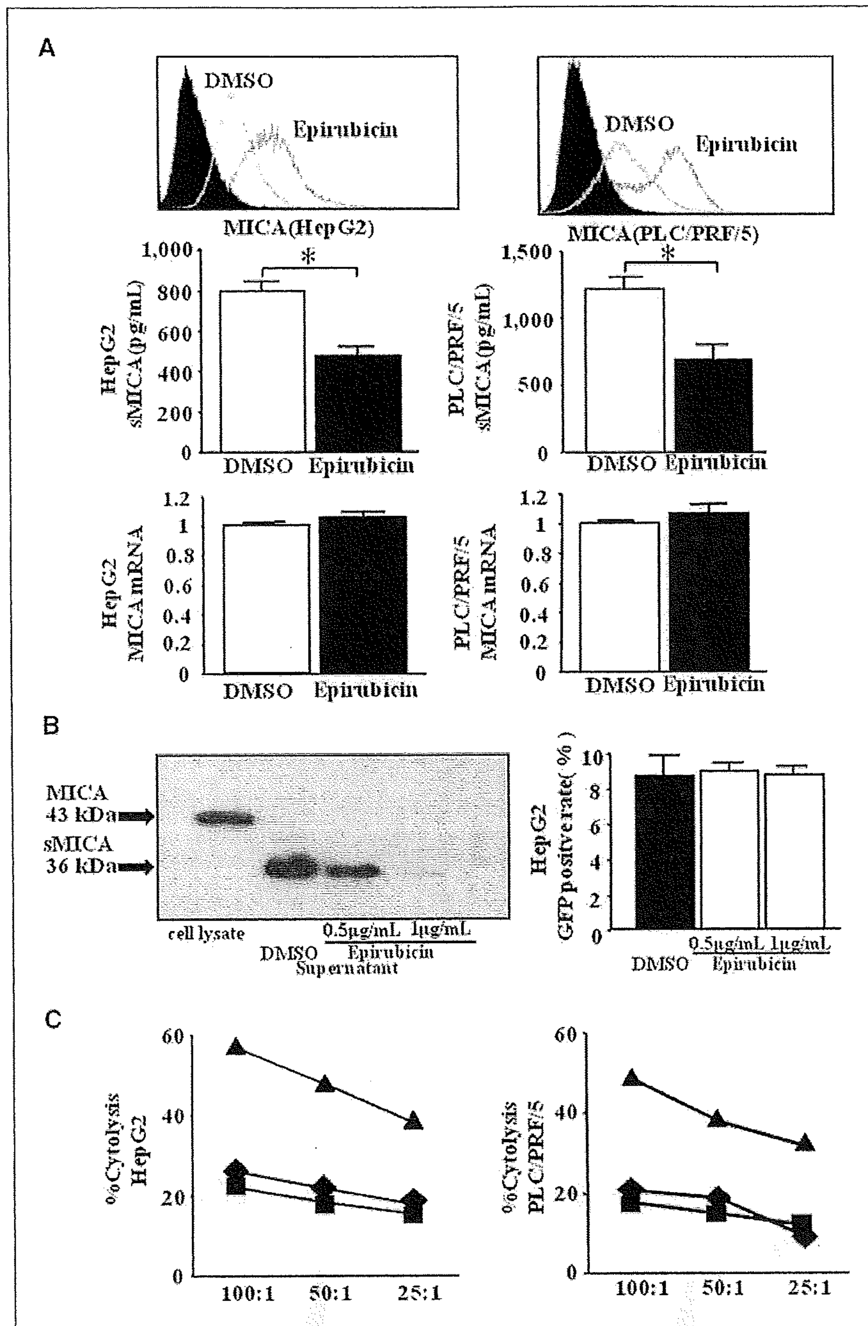

Figure 4. Expression and shedding of MICA in epirubicin-treated HCC cells. *A*, HCC cells were treated with a nontoxic dose of 1 μg/mL epirubicin (*black lines*) or vehicle (DMSO, *gray lines*) for 24 h and their expression of membrane-bound MICA and MICA mRNA was evaluated by flow cytometry (*top panels*) and real-time RT-PCR (*bottom panels*), respectively. *Closed histograms*, control IgG staining in flow cytometry. At the same time, 24-h culture supernatants were subjected to the analysis of soluble MICA (*sMICA*) levels by ELISA (*middle panels*). *, $P < 0.05$. *B*, HepG2 cells were transfected with pMyc-MICA and pEGFP-C1, cultured with 0.5 to 1 μg/mL epirubicin or vehicle (DMSO) for 24 h. Cell lysates from HepG2 cells and 24-h culture supernatants of epirubicin- or vehicle-treated HepG2 cells were immunoprecipitated with anti-Myc. The resulting immunoprecipitates were eluted, treated with N-glycanase, and subjected to Western blot analysis for MICA (*left*). Transfection efficacies were equal in all treatment groups as evidenced by similar GFP-positive cell rates (*right*). *C*, the cytolytic activity of NK cells against HCC cells. Vehicle-treated cells (■) or epirubicin-treated cells without (▲) or with blocking antibody of MICA/B (6D4; ♦) were subjected to ^{51}Cr-release assay. Representative results are shown. Similar results were obtained from three independent experiments.

had been tested with equal variance and Fisher's exact probability test. We defined statistical significance as $P < 0.05$.

Results

ADAM10 and CD44 are overexpressed in human HCC. ADAM10 was detected in all human HCC tissues tested by immunohistochemistry but not in normal liver tissues (Fig. 1*A*). Flow cytometric analysis revealed that ADAM10 was strongly expressed in a variety of HCC cell lines, including HepG2, PLC/PRF/5 (depicted in Fig. 1*B*), and Hep3B (data not shown), but faintly in primary hepatocytes. CD44, a typical substrate of the ADAM10 protease, was also expressed in all human HCC tissues but not in normal liver tissues (Fig. 1*A*). The data suggest that overexpression of ADAM10 and CD44 is a characteristic of human HCC like other malignancies (22).

ADAM10 is involved in MICA shedding of HCC cells but ADAM17 is not. To examine the involvement of ADAM family proteins in MICA ectodomain shedding, ADAM10 or ADAM17 were knocked down in HCC cells using a siRNA-mediated procedure. ADAM10 expression was clearly suppressed in HepG2 cells and PLC/PRF/5 cells at both mRNA and protein levels (Fig. 1*B* and *C*). Both cell lines expressed CD44 on the cellular surface and produced significant levels of soluble CD44 (Fig. 1*D*), indicating that CD44 is expressed and shed from those cell lines. ADAM10 knockdown (KD)

led to an increase in CD44 expression on HCC cells and a decrease in soluble CD44 levels in culture supernatants (Fig. 1*D*). Because ADAM10 has been established as being a sheddase for CD44, siRNA-mediated knockdown of ADAM10 suppressed not only the expression but also the activity of ADAM10 in HCC cells. HepG2 and PLC/PRF/5 cells also expressed ADAM17, which was clearly knocked down by a siRNA-mediated procedure (Fig. 1*B*).

HepG2 cells and PLC/PRF/5 cells expressed membrane-bound MICA and also produced soluble MICA (Fig. 2*A*). Knockdown of ADAM10 for both cell lines clearly upregulated MICA expression on their cellular surface and downregulated soluble MICA levels in their culture supernatant (Fig. 2*A*). In contrast, knockdown of ADAM17 did not affect the expression of membrane-bound MICA or the production of soluble MICA (Fig. 2*B*). We also examined the involvement of ADAM17 in MICA shedding of phorbol 12-myristate 13-acetate (PMA)–stimulated HCC cells because ADAM17 is considered to primarily affect stimulated shedding. The expression of membrane-bound MICA and the soluble MICA production were equal between PMA-stimulated ADAM17KD-HCC cells and control HCC cells (Supplementary Fig. S2). Thus, ADAM10, but not ADAM17, is critically involved in the shedding of MICA in HCC cells.

We next evaluated the cytolytic activity of NK cells against HCC cells. The cytolytic activity of NK cells against ADAM10KD-HepG2 cells was higher than that against control HepG2 cells. This activity was inhibited by blocking of anti-MICA/B antibody, suggesting that the increase of NK sensitivity depended on the increased expression of membrane-bound MICA on ADAM10KD-HepG2 cells, although we could not exclude the possibility of the involvement of MICB in this cytotoxicity (Fig. 2*C*). Similar results were also obtained with ADAM10KD-PLC/PRF/5 cells.

Epirubicin suppresses ADAM10 expression in HCC cells. We examined the biological modification of human HCC cells by adding epirubicin, which is commonly used in anti-HCC chemotherapy. We first examined the cytotoxicity of epirubicin to human HCC cells by WST-8 assay. Adding >5 μg/mL of epirubicin resulted in a significant decrease in cell growth of both HepG2 and PLC/PRF/5 cells (Fig. 3*B*). Based on these findings, we used 1 μg/mL of epirubicin to evaluate the biological effect on human HCC cells without toxicity. Both HepG2 cells and PLC/PRF/5 cells were cultured for 24 h with epirubicin and then subjected to analysis of ADAM10 expression. Epirubicin suppressed ADAM10 expression at the mRNA and protein levels in both cell lines (Fig. 3*C*). Although the data are not shown, doxorubicin also suppressed ADAM10 expression in HCC cells.

Epirubicin inhibits MICA ectodomain shedding and enhances susceptibility to NK cells of HCC cells. The above observations led us to investigate whether epirubicin or doxorubicin treatment would affect MICA ectodomain shedding in HCC cells. Epirubicin treatment led to an increase in membrane-bound MICA expression and a decrease in soluble MICA production in both HepG2 and PLC/PRF/5 cells (Fig. 4*A*). The mRNA levels of MICA did not change after exposure to epirubicin in both HCC cells (Fig. 4*A*). Similar data were obtained with doxorubicin-treated cells (data not shown).

To confirm whether the soluble MICA detected by ELISA was actually reflected in the cleaved form, we transfected Myc-tagged MICA into HepG2 cells and collected culture supernatants as well as cellular lysates. Immunprecipitates from these samples with anti-Myc were subjected to Western blot analysis after treatment with N-glycosidase. MICA in the culture supernatants migrated faster than cellular MICA (Fig. 4*B*), indicating that the MICA detected by ELISA is actually processed and released from full-length MICA. Epirubicin treatment led to a decrease in soluble MICA protein in HepG2 cells (Fig. 4*B*).

We next evaluated whether the epirubicin treatment could also modify the NK sensitivity of human HCC cells. Epirubicin-treated HepG2 cells or PLC/PRF/5 cells were more susceptible to NK cells than nontreated HepG2 or PLC/PRF/5 cells (Fig. 4*C*). The cytolytic activity against epirubicin-treated HCC cells was significantly decreased to the control levels by adding the anti-MICA/B blocking antibody. These results showed that the addition of epirubicin enhanced the NK sensitivity of HCC cell through increased

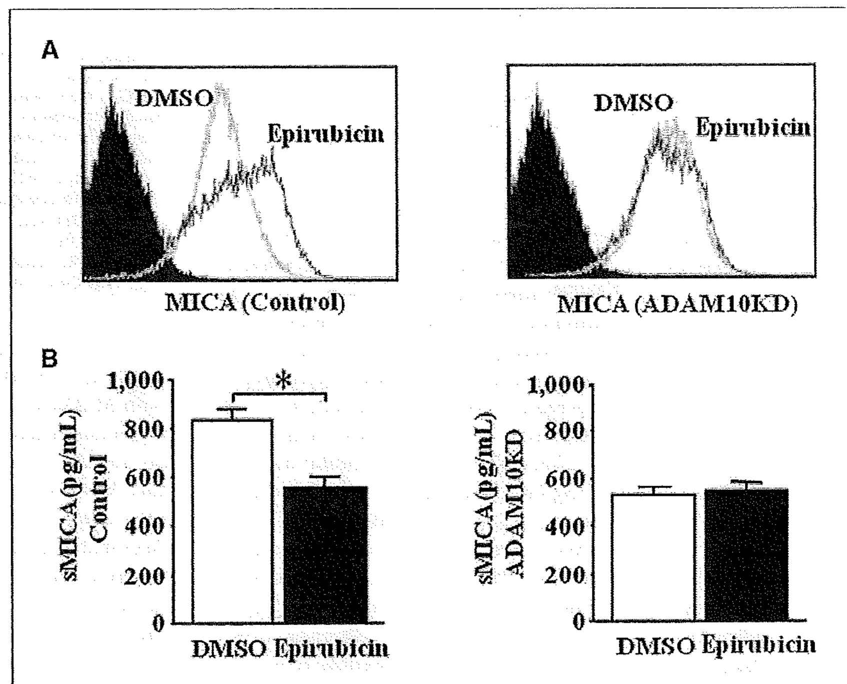

Figure 5. The epirubicin-mediated modification of MICA is ADAM10 dependent. HepG2 cells were transfected with ADAM10 siRNA (*ADAM10KD*) or control siRNA (*Control*) and further cultured with 1 μg/mL of epirubicin (*black lines*) or vehicle (DMSO, *gray line*) for 24 h. The expression of membrane-bound MICA (*MICA*) was evaluated by flow cytometry (*A*), and the soluble MICA (*sMICA*) production in the culture supernatant was evaluated by specific ELISA (*B*). Similar results were obtained from two independent experiments. *, $P < 0.05$.

expression of membrane-bound MICA, although the possibility of MICB involvement could not be excluded. The doxorubicin-treated human HCC cells showed similar results to those obtained from epirubicin-treated HCC cells (data not shown).

Epirubicin inhibits MICA ectodomain shedding through suppression of ADAM10. To examine whether the suppressive effect of epirubicin on MICA shedding occurred through downregulation of ADAM10, HepG2 cells were transfected with ADAM10 siRNA or scramble siRNA as a control and then treated with epirubicin. Consistent with earlier observations, epirubicin upregulated MICA surface expression and downregulated the levels of soluble MICA in control cells (Fig. 5). In contrast, neither upregulation of surface MICA nor downregulation of soluble MICA levels was observed in ADAM10KD-HepG2 cells. These results suggest that the suppressive effect of epirubicin on MICA shedding is mediated by ADAM10 downregulation. We also found similar results with ADAM10KD-PLC/PRF/5 cells (data not shown).

Soluble CD44 and soluble MICA levels in patients with HCC. We have shown that ADAM10 is expressed in human HCC tissues. However, it is not clear whether ADAM10 activity in HCC tissues is actually involved in MICA shedding in patients. Because ADAM10 was reported to be the constitutive functional sheddase of CD44 (23), we examined the soluble CD44 levels in HCC patients, which might be produced from tumor cells through ADAM10 activity. As shown in Fig. 6*A*, the soluble CD44 levels in HCC patients (n = 97) were significantly higher than those in age-matched healthy volunteers (n = 32). More importantly, soluble MICA levels in HCC patients significantly correlated with soluble CD44 levels (Fig. 6*B*), suggesting a close link between MICA shedding and ADAM10 activity.

We further examined soluble CD44 levels before and 2 weeks after TACE in HCC patients. Whereas the levels did not change in nontreated HCC patients during the 2-week interval (n = 9; 306.7 ± 82.5 ng/mL and 309.9 ± 79.9 ng/mL after 2 weeks), they were significantly decreased in epirubicin-based TACE-treated HCC patients (n = 21; 339.7 ± 78.1 ng/mL before TACE and 308.9 ± 81.4 ng/mL after TACE, $P < 0.003$). The changes of soluble CD44 in TACE treatment correlated significantly with those of soluble MICA ($P = 0.0002$; Fig. 6*C*). These results indicated that ADAM10-mediated CD44 shedding was decreased after TACE in HCC patients, implying that this reduction of ADAM10 activity might be related to the decline in MICA shedding.

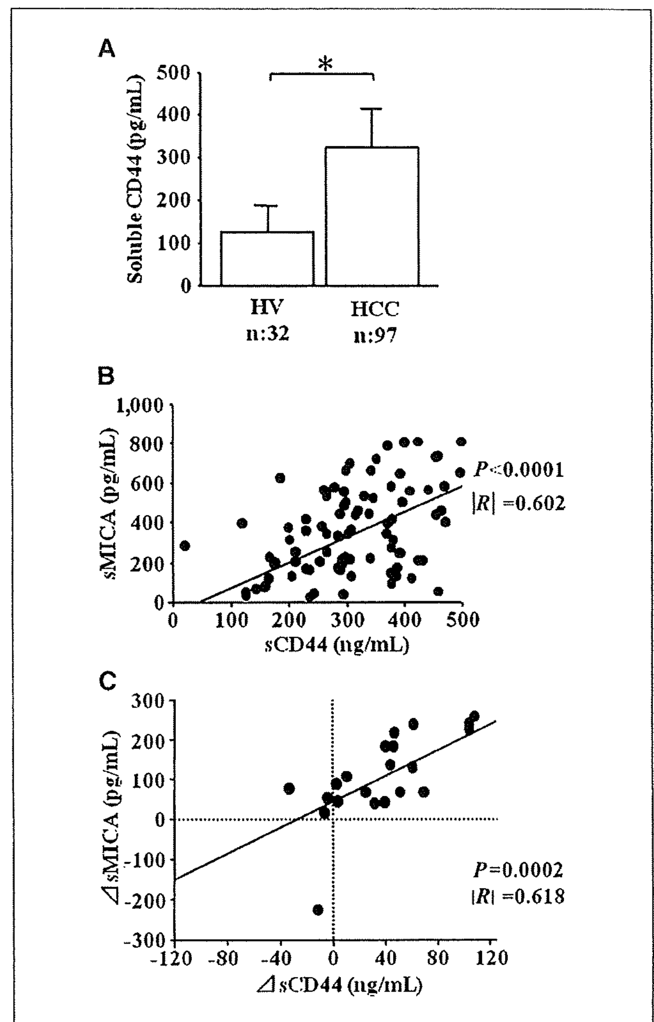

Figure 6. Correlation between soluble CD44 and soluble MICA in human HCC patients. *A* and *B*, soluble CD44 levels and MICA levels in healthy volunteers and HCC patients. Soluble CD44 levels (*sCD44*) and soluble MICA levels (*sMICA*) were determined for sera of HCC patients (n = 97) and age-matched healthy volunteers (*HV*; n = 32). *A*, comparison of sCD44 levels between groups; *B*, correlation between sCD44 levels and sMICA levels in 97 HCC patients. *, $P < 0.05$. *C*, correlation of sCD44 levels and sMICA levels during TACE therapy. HCC patients (n = 21) treated with epirubicin-based TACE therapy were enrolled and examined for sMICA and sCD44 levels before and 2 wk after therapy. Changes in sMICA (ΔsMICA = serum level of sMICA before TACE treatment − serum level of sMICA after TACE treatment) and those in sCD44 levels (ΔsCD44 = serum level of sCD44 before TACE treatment − serum level of sCD44 after TACE treatment) are plotted.

Discussion

MICA shedding is thought to be a principal mechanism by which tumor cells escape from NKG2D-mediated immunosurveillance (13). Thus, inhibition of MICA shedding should be a reasonable strategy for enhancing antitumor immunity. In the present study, we showed that ADAM10 was overexpressed in human HCC tissues and that ADAM10 knockdown resulted in increased expression of membrane-bound MICA, decreased production of soluble MICA, and upregulation of NK sensitivity of human HCC cells. These results point to ADAM10 as a therapeutic target for inhibiting MICA shedding, thereby ameliorating immunity against HCC. Waldhauer and colleagues recently showed that both ADAM10 and ADAM17 proteases are critically involved in the proteolytic release of soluble MICA of human 293T fibroblast cells and HeLa cervix carcinoma cells (20). Interestingly, in the present study, ADAM17 knockdown failed to affect MICA expression in human HepG2 cells or PLC/PRF/5 cells. Thus, ADAM10, not ADAM17, plays an essential role in the shedding of MICA in human HCC cells. Anderegg and colleagues (23) reported that only ADAM10, not ADAM17, contributed to shedding of CD44 molecules in human melanoma cells although both ADAM10 and ADAM17 proteases were significantly expressed in human melanoma tissues, suggesting that ADAM10 and ADAM17 do not always work in a similar manner. A recent report showed that ADAM10, but not ADAM17, could directly bind to calmodulin (24), which may involve the difference of MICA cleavage between ADAM10 and ADAM17 proteases. Recently, Boutet and colleagues reported that ADAM17 regulates proteolytic shedding of the MICB protein, which is another ligand for the NKG2D receptor on immune cells (25). We previously showed that both soluble MICA and MICB significantly increased in the sera of HCC patients and that therapeutic intervention for HCC leads to reduction of soluble

MICA levels, but not of soluble MICB levels (17), suggesting a more important role of soluble MICA in regulating NKG2D expression after HCC therapy. This led us to focus on the mechanism of MICA shedding in the present study.

Our results revealed that anticancer drugs such as epirubicin and doxorubicin downregulated ADAM10 expression and activity, thereby inhibiting MICA ectodomain shedding. The ADAM family proteins, which are highly expressed in some tumors, play a role in secreting growth factors, such as HB-EGF, and migration of cells. Thus, it is speculated that these proteins could be potential targets for tumor treatment (22). The present study is the first to show that clinically available anticancer drugs have an ability to modulate the expression of ADAM family proteins. They seem to suppress ADAM10 expression at a transcriptional level, but the precise mechanism of this suppression is not yet known.

The MICA ELISA system may not equally detect all soluble MICA (MICA molecules have >60 allelic variants). Our finding that soluble MICA could be detected in all HCC patients suggests that this system was applicable for our cohort of HCC patients. However, special caution should be paid for the use of this ELISA system for widely polymorphic MICA. Because CD44 is well known to be released into circulation from tumors by proteolytic cleavage of ADAM10 (23), the activity of ADAM10 in HCC tissues may be correlated with soluble CD44 levels. If so, our data suggest a close link between ADAM10 activity and the shedding of MICA in HCC. Furthermore, the decline in soluble MICA levels correlated well with the decline in soluble CD44 levels as early as 2 weeks after epirubicin-based TACE therapy. Reducing the tumor volume by such therapy may have led to both decreases but it is also possible that epirubicin suppresses ADAM10 activity, thereby inhibiting the shedding of MICA and CD44. Epirubicin may have a previously unrecognized role in cancer therapy; that is, affecting ADAM10 activity and MICA shedding rather than simply serving as a direct toxic agent for tumor cells.

Our data suggest that anti-HCC chemotherapy could remodel HCC cells, enhancing sensitivity to NK cells by upregulating MICA expression on the cellular surface. A concomitant decline in soluble MICA levels ameliorates NK cell ability by upregulating its NKG2D expression. We previously showed that activation of local innate antitumor immunity in liver tissues resulted in eliciting tumor-specific acquired immunity (21). If liver innate immunity is efficiently activated after anti-HCC chemotherapy, an additional antitumor effect against HCC cells could be expected. Immune modulators such as α-galactosylceramide have been shown to efficiently activate liver innate immune cells, including NK cells (21, 26). The combination therapy of anti-HCC chemotherapy and immunotherapy targeting NK cells might improve the antitumor effect of unresectable HCC and the prognosis of HCC patients.

In spite of recent progress in HCC therapies, there remains significant room for improvement, especially with respect to advanced liver cancer. We have shown here that anti-HCC chemotherapy resulted in enhanced NK sensitivity of HCC cells through inhibition of the activity of ADAM10 protease followed by modification of MICA expression. These findings indicate that efficient activation of liver innate immunity after anti-HCC chemotherapy might represent a particularly promising approach to suppress tumor growth and promote regression in liver cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/4/09; revised 7/15/09; accepted 7/24/09; published OnlineFirst 10/13/09.

Grant support: Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (T. Takehara) and Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan (N. Hayashi).

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Factors affecting efficacy in patients with genotype 2 chronic hepatitis C treated by pegylated interferon alpha-2b and ribavirin: reducing drug doses has no impact on rapid and sustained virological responses

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Received May 2009; accepted for publication June 2009

SUMMARY. Reducing the dose of drug affects treatment efficacy in pegylated interferon (Peg-IFN) and ribavirin combination therapy for patients with hepatitis C virus (HCV) genotype 1. The aim of this study was to investigate the impact of drug exposure, as well as the baseline factors and the virological response on the treatment efficacy for genotype 2 patients. Two-hundred and fifty patients with genotype 2 HCV who were to undergo combination therapy for 24 weeks were included in the study, and 213 completed the treatment. Significantly more patients who achieved a rapid virological response (RVR), defined as HCV RNA negativity at week 4, achieved a sustained virological response (SVR) (92%, 122/133) compared with patients who failed to achieve RVR (48%, 38/80) ($P < 0.0001$). Multivariate logistic-regression analysis showed that only platelet counts [odds ratio (OR), 1.68; confidence interval (CI), 1.002–1.139] and RVR (OR, 11.251; CI, 5.184–24.419) were independently associated with SVR, with no correlation being found for the mean dose of Peg-IFN and ribavirin for RVR and SVR. Furthermore, in the stratification analysis of the timing of viral clearance, neither mean dose of Peg-IFN ($P = 0.795$) nor ribavirin ($P = 0.649$) affected SVR in each group. Among the patients with RVR, the lowest dose group of Peg-IFN (0.77 ± 0.10 μg/kg/week) and ribavirin (6.9 ± 0.90 mg/kg/day) showed 100% and 94% of SVR. Hence, RVR served as an important treatment predictor, and drug exposure had no impact on both SVR and RVR in combination therapy for genotype 2 patients.

Keywords: chronic hepatitis C, drug exposure, genotype 2, peginterferon and ribavirin combination therapy.

INTRODUCTION

The current standard of care for chronic hepatitis C (CHC) patients consists of combination therapy using pegylated interferon (Peg-IFN) and ribavirin [1–3]. Large, randomized clinical trials have demonstrated that 42–52% of hepatitis C virus (HCV) genotype 1 'difficult-to-treat' patients achieved sustained virological response (SVR), whereas 76–84% of HCV genotype 2 or 3 infected patients treated with Peg-IFN and ribavirin achieved SVR [4–6]. It also has been shown that in HCV genotype 2 and 3 infected patients, 24-week treatment regimens are just as effective as 48-week regimens [6,7]. Therefore, current guidelines recommend a 24-week treatment for these patients in contrast to 48 weeks for genotype 1 patients [1–3]. However, as side effects are common and treatment is expensive for this therapy, it would be ideal to be able to further reduce the total amount of drug medication

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; c-EVR, complete early virological response; ETR, end of treatment response; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; NPV, negative predictive value; Peg-IFN, pegylated interferon; RVR, rapid virological response; SVR, sustained virological response.

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without loss of treatment efficacy for genotype 2 and 3 patients.

In HCV genotype 1 patients, reducing drug doses affects treatment efficacy. In our investigation of HCV genotype 1 patients, the rate of complete early virological response (c-EVR), defined as HCV RNA negativity at week 12, was affected by the mean dose of Peg-IFN during the first 12 weeks dose-dependently ($P < 0.0001$) [8]. Furthermore, we showed that only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin among those with c-EVR, and the relapse rate showed a decline in relation to the increase in the dose of ribavirin ($P = 0.0002$) [9]. On the contrary, it remains to be determined whether treatment efficacy can be preserved by further reducing both drug doses in genotype 2 and 3 patients. Because lower doses are expected to cause fewer adverse effects, it is important to find whether reduced drug doses can be used while retaining efficacy.

In the present study, we retrospectively evaluated the efficacy of Peg-IFN alpha-2b and ribavirin combination therapy for 24 weeks in patients infected with HCV genotype 2 and analysed the factors that affected the treatment efficacy, with particular interests in the drug impact of Peg-IFN and ribavirin.

PATIENTS AND METHODS

Patient selection and study design

Patients considered to be eligible for this study were those infected with HCV genotype 2 who underwent Peg-IFN alpha-2b (Schering-Plough K.K., Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to July 2007 at 29 medical institutions taking part in the Osaka Liver Forum and had completed the 24-week observation after a clinical course of 24 weeks. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus coinfection, decompensated liver disease, severe cardiac, renal, haematological or chronic pulmonary disease, poorly controlled psychiatric disease, poorly controlled diabetes and immunologically mediated disease. Liver biopsy had been performed within 24 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [10].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by institutional review boards at the respective sites.

Patients were treated with Peg-IFN alpha-2b plus ribavirin for the duration of the study of 24 weeks. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; 91 kg or more, 150 μg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; 81 kg or more, 1000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the intensity of the haematologic adverse effects.

Virological tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5000 IU/mL and a dynamic range from 5000 to 5 000 000 IU/mL [11].

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/mL [12].

Assessment of efficacy

Serum HCV RNA (qualitatively or quantitatively) was measured at weeks 4, 8, 12 and 24 during treatment and after 24 weeks of follow-up without treatment. Patients were classified as having a rapid virological response (RVR) if serum HCV RNA was undetectable (<50 IU/mL) at week 4 and at the end of treatment response (ETR) at week 24 of treatment. SVR was defined as undetectable HCV RNA at week 24 after treatment. Patients with an ETR who sero-reverted to HCV RNA during follow-up were classified as relapsers.

Drug exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as μg/kg/week and ribavirin as mg/kg/day.

Data collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

Statistical analysis

This study was a retrospective study and, for treatment results and the analysis of related factors, analysis was carried out only for cases in which the treatment had been completed (per-protocol analysis). Continuous variables are reported as the mean with standard deviation (SD) or

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median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann–Whitney *U*-test was used to analyse continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. Variables with $P < 0.05$ at univariate analysis were retained for the multivariate logistic-regression analysis. Stepwise and multivariate logistic-regression models were used to explore the independent factors that could be used to predict a virological response. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant if $P < 0.05$. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL, USA).

RESULTS

The baseline characteristics for the total cohort are shown in Table 1. Most of the patients were female (56%) with a mean age of 54 years. Seventy per cent of the patients were treatment naïve. Of the 250 patients, liver biopsies were performed for 174 patients, and 18 of them had advanced fibrosis (F 3–4).

Of the total of 250 patients, 37 (15%) were withdrawn from treatment because of adverse events: decreased haemoglobin (n = 10), psychiatric problems including depression (n = 9), fatigue (n = 3), thrombocytopenia, neutropenia, pyrexia, rash, cerebral haemorrhage, bleeding of ocular fundus, dyspnea, dizziness, jaundice, transaminase rise, gastrointestinal symptoms (n = 1) and other adverse events (n = 4). Eight of these patients who discontinued treatment prematurely had SVR (8/37; 22%).

Drug adherence

Seventy-nine of the 213 patients (37%) required dose reduction of Peg-IFN alpha-2b, 99 (46%) of ribavirin because of adverse events (not including patients who later discontinued treatment because of adverse event). Neutropenia (24/79; 30%) and thrombocytopenia (24/79; 30%) were the most common adverse events for dose reduction of Peg-IFN alpha-2b, and decreased haemoglobin (82/99; 83%) for that of ribavirin.

Virological response

Of the 213 patients who completed 24 weeks of treatment and 24 weeks of follow-up, 160 (75%) patients were clear of HCV RNA at week 4, 191 (90%) at week 8, 196 (92%) at week 12. ETR was observed for 195 (92%), and SVR for 160 (75%). The relapse rate was 18% (35/195).

Virological response according to the timing of viral clearance

Positive and negative prediction of sustained virological response according to the timing of viral clearance

We examined SVR rates according to the timing of viral clearance for the case in which HCV RNA was cleared during the treatment (Fig. 1a). The SVR rate was 92% (122/133) for patients clear of HCV RNA until week 4, 64% (37/58) from week 5 until week 8, 20% (1/5) from week 9 until

Table 1 Baseline demographic and viral characteristics of patients

Number of cases	250	
Age (years)*	54.0 ± 12.4	(22–76)
Sex (male/female)	110/140	
Body weight (kg)*	60.3 ± 11.7	(39–99)
Body mass index (kg/m^2)*	23.1 ± 3.2	(16–35)
Past IFN therapy (naïve/experienced)†	175/70	
HCV RNA (KIU/mL)‡	1700	(4–5000 <)
Fibrosis (0/1/2/3/4)§	18/98/40/14/4	
Activity (0/1/2/3)§	15/81/70/8	
White blood cells (/mm^3)*	5210 ± 1,750	(2100–13 870)
Neutrophils (/mm^3)*	2700 ± 1,250	(590–9020)
Red blood cells (×10^4/mm^3)*	436 ± 48	(307–554)
Haemoglobin (g/dL)*	13.9 ± 1.4	(10–18)
Platelets (×10^4/mm^3)*	18.3 ± 6.4	(4–41)
ALT (IU/L)*	79 ± 77	(13–581)
γ-GTP (U/L)*	56 ± 65	(7–479)
Creatinine(mg/dL)*	0.7 ± 0.1	(0.4–1.1)

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase. *Values expressed as mean ± SD (range), †interferon treatment history was not known for five patients, ‡values expressed as median (range), §data for 76 patients are missing.

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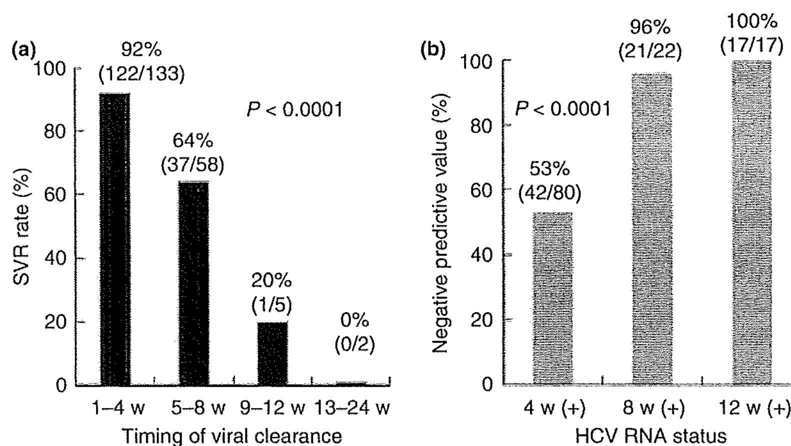

Fig. 1 (a) SVR rates according to timing of viral clearance. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The timing of viral clearance was time-dependently correlated with SVR ($P < 0.0001$). (b) Negative predictive values according to time of HCV RNA positivity. The number above each bar shows the percentage, and the numbers inside parentheses show the number of patients showing responses over the total number in the subgroup. The time of HCV RNA positivity was time-dependently correlated with NPV ($P < 0.0001$).

week 12 and 0% (0/2) from week 13 until week 24. The Mantel–Haenszel chi-square test showed that SVR rates were diminished with a delay in the timing of viral clearance becoming late ($P < 0.0001$). Significantly, more patients who attained RVR achieved final SVR (92%, 122/133) than patients who failed to attain RVR (48%, 38/80; $P < 0.0001$).

Next, we examined the negative predictive value (NPV) for the proportion of patients with treatment failure among those with HCV RNA persistence at week 4, 8 and 12 (Fig. 1b). NPV was 53% at week 4, 96% at week 8 and 100% at week 12. Only one of the 22 patients with positive HCV RNA at week 8 reached SVR.

Predictors of sustained virological response

Both pretreatment and treatment factors that could be associated with the response to Peg-IFN and ribavirin combination therapy were compared between patients with and without SVR in Table 2. This univariate analysis showed that age ($P = 0.029$), baseline HCV RNA level ($P = 0.033$), past IFN treatment history ($P = 0.028$), platelets counts ($P = 0.020$) and having RVR ($P < 0.0001$) contributed to achievement of SVR. Factors that were significantly associated with SVR by univariate analysis were then analysed by multivariate logistic regression analysis. SVR was attained independent of high platelet counts [odds ratio (OR) 1.070, 95% confidence interval (CI) 1.003–1.140, $P = 0.040$] and having RVR (OR 11.526, 95% CI 5.317–24.984, $P < 0.0001$; Table 3). As for drug doses, the mean dose of Peg-IFN alpha-2b was 1.32 ± 0.27 μg/kg/week in patients with SVR and 1.27 ± 0.29 μg/kg/week in those without SVR ($P = 0.130$), while that of ribavirin was 10.2 ± 1.9 and 10.2 ± 2.0 mg/kg/day ($P = 0.949$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the full treatment period affected attainment of SVR.

Predictors of rapid virological response

To delineate features that might help identify patients most likely to reach RVR, we also analysed these factors because having RVR turned out to be one of the most powerful predictors of SVR attainment. By univariate and multivariate logistic-regression analyses, RVR was attained independent of younger age (OR 0.648, 95% CI 0.494–0.850, $P = 0.002$) and lower baseline HCV RNA level (OR 0.964, 95% CI 0.944–0.984, $P < 0.0001$; Tables 4 & 5). The mean dose of Peg-IFN alpha-2b during the first 4 weeks was 1.31 ± 0.27 μg/kg/week in patients with RVR and 1.31 ± 0.29 μg/kg/week in those without RVR ($P = 0.259$), that of ribavirin was 10.1 ± 1.8 mg/kg/day and 10.3 ± 2.1 mg/kg/day ($P = 0.637$), respectively. Thus, neither Peg-IFN nor ribavirin drug exposure during the first 4 weeks had an impact on attainment of RVR.

Virological response according to drug exposure and the timing of viral clearance

Impact of drug exposure on sustained virological response

To more closely evaluate the impact of drug exposure on virological response, we classified the average doses of both drugs into four categories (Peg-IFN alpha-2b: up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week, from 1.5 μg/kg/week; ribavirin: up to

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Table 2 Factors associated with SVR among patients who completed the treatment – univariate analysis

Factor	SVR (n = 160)	Non-SVR (n = 53)	P-value
Age (years)*	52.4 ± 12.6	56.9 ± 10.2	0.029
Sex (male/female)	66 / 94	26 / 27	0.202
Body weight (kg)*	59.5 ± 11.5	59.9 ± 12.5	0.896
Body mass index (kg/m^2)*	22.8 ± 3.1	22.8 ± 3.5	0.817
HCV RNA (KIU/mL)†	1170	1600	0.033
Past IFN therapy (naive/experienced)‡	116/41	31/22	0.028
Fibrosis (F 0–2/3–4)§	106/10	30/5	0.247
Activity (A 0–1/2–3)§	62/54	20/15	0.847
White blood cells (/mm^3)*	5260 ± 1680	4720 ± 1500	0.078
Neutrophils (/mm^3)*	2740 ± 1270	2420 ± 1020	0.186
Red blood cells (×10^4/mm^3)*	435 ± 44	437 ± 55	0.820
Haemoglobin (g/dL)*	13.9 ± 1.3	14.0 ± 1.5	0.441
Platelets (×10^4/mm^3)*	19.0 ± 6.0	16.5 ± 6.2	0.020
ALT (IU/L)*	86 ± 89	64 ± 45	0.514
γ-GTP (U/L)*	54 ± 67	58 ± 59	0.512
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.457
Mean Peg-IFN dose (μg/kg/week)*	1.32 ± 0.27	1.27 ± 0.29	0.130
Mean ribavirin dose (mg/kg/day)*	10.2 ± 1.9	10.2 ± 2.0	0.949
RVR (yes/no)	122/11	38/42	<0.0001

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± sd, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 3 Factors associated with SVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	–	–	NS
HCV RNA (KIU/mL)	By 100 KIU/mL	–	–	NS
Platelets (×10^4/mm^3)	By 1 × 10^4/mm^3	1.068	1.002–1.139	0.045
Past IFN therapy	Naïve/experienced	–	–	NS
RVR	Yes/no	11.251	5.184–24.419	<0.0001

IFN, interferon; HCV, hepatitis C virus; CI, confidence interval.

8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day, from 12 mg/kg/day). SVR rates relative to the mean drug doses during the full treatment period and the timing of HCV RNA clearance are shown in Table 6. As also shown in Fig. 1a, the respective rates for SVR according to the timing of viral clearance were 92% in patients clear of HCV RNA until week 4, 64% from week 5 until week 8 and 14% from week 9 until week 24. On the contrary, according to mean drug doses, the respective rates for SVR were 89% (24/27), 73% (11/15), 79% (85/107) and 82% (40/49) in patients who received Peg-IFN up to 0.9 μg/kg/week, from 0.9 to >1.2 μg/kg/week, from 1.2 to >1.5 μg/kg/week and from 1.5 μg/kg/week, respectively, and 80% (24/30), 80% (40/50), 82% (68/83) and 79% (27/34) in patients who received ribavirin up to 8 mg/kg/day, from 8 to >10 mg/kg/day, from 10 to >12 mg/kg/day and from 12 mg/kg/day, respectively. If the category of the timing of viral clearance was the same, the respective rates for SVR attainment according to the mean doses of both Peg-IFN and ribavirin were similar. Furthermore, multivariate analysis by the Mantel–Haenszel chi-square test showed that neither the mean dose of Peg-IFN (P = 0.795) nor ribavirin (P = 0.649) affected SVR rates after stratification of the timing of viral clearance. Among the patients with RVR, SVR rates were as high as 88–100% regardless of Peg-IFN alpha-2b medication, and the least medicated group (<0.9 μg/kg/week, the mean dose with SD was 0.77 ± 0.10 μg/kg/week, 0.50–0.89) showed 100% of SVR rate (19/19). Similarly, SVR rates were as high as 91–94% regardless of ribavirin medication among the patients with RVR, and 17 of 18 patients (94%) in the least medicated group (<8 mg/kg/day, the mean dose with SD was 6.9 ± 0.90 mg/kg/day, 5.0–7.9)

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Table 4 Factors associated with RVR among patients who completed the treatment – univariate analysis

Factor	RVR (n = 133)	Non-RVR (n = 80)	P-value
Age (years)*	51.9 ± 12.3	56.3 ± 11.3	0.010
Sex (male/female)	60/73	32/48	0.279
Body weight (kg)*	60.2 ± 11.6	58.6 ± 11.9	0.276
Body mass index (kg/m^2)*	22.9 ± 3.2	22.6 ± 3.1	0.369
HCV RNA (KIU/mL)†	1050	1800	0.001
Past IFN therapy (naive/experienced)‡	97/34	50/29	0.068
Fibrosis (F 0–2/3–4)§	86/8	50/7	0.315
Activity (A 0–1/2–3)§	51/43	31/26	1.000
White blood cells (per mm^3)*	5300 ± 1760	4850 ± 1400	0.205
Neutrophils (per mm^3)*	2740 ± 1290	2530 ± 1090	0.340
Red blood cells (×10^4/mm^3)*	440 ± 45	432 ± 49	0.628
Haemoglobin (g/dL)*	13.9 ± 1.4	13.9 ± 1.4	0.975
Platelets (×10^4/mm^3)*	18.9 ± 6.1	17.5 ± 6.1	0.170
ALT (IU/L)*	87 ± 93	69 ± 52	0.630
γ-GTP (U/L)*	57 ± 71	53 ± 53	0.658
Creatinine (mg/dL)*	0.7 ± 0.1	0.7 ± 0.1	0.203
Mean Peg-IFN dose (μg/kg/week)*	1.31 ± 0.27	1.31 ± 0.29	0.259
Mean ribavirin dose (mg/kg/day)*	10.1 ± 1.8	10.3 ± 2.1	0.637

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; CI, confidence interval. *Values expressed as mean ± SD, †values expressed as median, ‡interferon treatment history was not known for three patients, §data for 62 patients are missing.

Table 5 Factors associated with RVR among patients who completed the treatment – multivariate analysis

Factor	Category	Odds ratio	95% CI	P-value
Age (years)	By 10	0.648	0.494–0.850	0.002
HCV RNA (KIU/mL)	By 100 KIU/mL	0.964	0.944–0.984	<0.0001

HCV, hepatitis C virus; CI, confidence interval.

achieved SVR. In addition, we examined the drug impact on SVR in the patients with the least medication of both drugs (<0.9 μg/kg/week of Peg-IFN and <8 mg/kg/day of ribavirin). Nine patients were categorized into this group and six of these patients achieved SVR (67%); patients with RVR had a significantly higher SVR rate (100%, 5/5) than patients without RVR (25%, 1/4; P = 0.048). Thus, SVR attainment was dependent on time, not on drug dose.

DISCUSSION

In the present study, we found that having RVR and high platelet counts were statistically associated with reaching SVR according to multivariate analysis. The timing of viral clearance was closely related to the treatment effect in patients with genotype 2, similar to the case for those with genotype 1. Ninety-two per cent of SVR was observed for patients with RVR and, conversely, 96% of the patients with HCV RNA positivity at week 8 showed non-SVR. The predictability of SVR based on EVR, defined as a decline of at least 2-log from the baseline of the HCV RNA level at week 12, has been assessed, and genotype 1 patients who have failed to reach EVR are recommended to discontinue the treatment after 12 weeks, because the likelihood of SVR is 0–3% in the absence of EVR [5,13]. On the basis of our examination of patients with genotype 2, not EVR, but 8-week monitoring of the HCV RNA level can be used.

As a significant factor for SVR, not liver fibrosis, but the platelet count was selected. Everson *et al.* [14] reported that patients with low platelet counts (≤12.5 × 10^4/mm^3) achieved lower SVR rates than patients with normal platelet counts (>12.5 × 10^4/mm^3) even in the case of patients with the same category of liver fibrosis treated by Peg-IFN plus ribavirin combination therapy. Thus, independent of liver fibrosis, thrombocytopenia itself seems to participate in treatment failure, although the mechanism remains unknown.

Our study also demonstrated that younger age (OR 0.648, 95% CI 0.494–0.850, P = 0.002) and lower HCV RNA level (OR 0.964, 95% CI 0.944–0.984, P < 0.0001) were statistically associated with reaching an RVR. Zeuzem *et al.* [7] previously reported that pretreatment viral load was not

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Table 6 SVR rates according to Peg-IFN alpha-2b and ribavirin exposure and the timing of viral clearance among patients with virological response during the treatment

Timing of viral clearance (week)	Peg-IFN dose (μg/kg/week)				Ribavirin dose (mg/kg/day)				Total
	<0.9	0.9–1.2	1.2–1.5	1.5≤	<8	8–10	10–12	12≤	
1–4	100% (19/19)	91% (10/11)	92% (65/71)	88% (28/32)	94% (17/18)	92% (33/36)	91% (51/56)	91% (20/22)	92% (122/133)
5–8	63% (5/8)	33% (1/3)	64% (19/30)	71% (12/17)	58% (7/12)	54% (7/13)	74% (17/23)	60% (6/10)	64% (37/58)
9–24	–	0% (0/1)	17% (1/6)	–	–	0% (0/1)	0% (0/4)	50% (1/2)	14% (1/7)
Total	89% (24/27)	73% (11/15)	79% (85/107)	82% (40/49)	80% (24/30)	80% (40/50)	82% (68/83)	79% (27/34)	81% (160/198)

*P = 0.795 for comparison of the four Peg-IFN groups after stratification of the timing of viral clearance. **P = 0.649 for comparison of the four ribavirin groups after stratification of the timing of viral clearance.

associated with reaching RVR in genotype 2 patients. In contrast, Dalgard *et al.* [15] reported that independent predictors of RVR in genotype 2 or 3 patients were male gender, younger age (≤40 years) and low viral load (≤400/KIU/mL). The influence of viral load on reaching RVR remains controversial in the Peg-IFN and ribavirin combination therapy in genotype 2 patients, but patients with lower viral load seem favoured to reach HCV RNA levels below the detection limit, that is, to attain RVR, if the virological response is the same.

Recently, because of substantial adverse effects and costs associated with this therapy, studies have been carried out to determine the possibility of further reducing the total amount of drug medication without compromising antiviral efficacy in HCV genotype 2 and 3 patients. There seem to be two ways to achieve. One is by shortening the treatment duration, and the other is by decreasing the doses of the treatment drugs. With respect to the former, several studies on genotype 2 patients have been reported. At first, some studies of small numbers of subjects demonstrated that cumulatively analysed genotype 2 and 3 patients had high SVR rates up to 12 to 16 weeks of therapy (82–94%), similar to patients subjected to 24-week therapy (76–95%) [16–19]. However, further prospective investigation of large numbers of subjects revealed that shortening the treatment duration was associated with an increase in the rate of relapse and that significantly higher relapse rates led to lower SVR rates (71–81.1%), even among those with RVR [15,20,21]. The latest study by Mangia *et al.* [22] showed that shortened therapy after RVR was acceptable only for patients who had no signs of advanced liver fibrosis and low BMI. Considering the results of these trials, shortened therapy is regarded as optional treatment for selected patients displaying favourable baseline characteristics. Therefore, shortening treatment duration from 24 weeks should not be generally recommended for patients who are infected genotype 2 or 3 and can tolerate 24-week Peg-IFN and ribavirin combination therapy.

Another attempt to improve the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland *et al.* [23] examined low-dose Peg-IFN alpha-2a (135 μg weekly) with a weight-based standard-dose of ribavirin (11 mg/kg daily) for genotype 2 and 3 patients. They demonstrated that SVR rates of 86% were achieved, which is equal to those in previous representative randomized controlled studies of standard dose Peg-IFN therapy (76–84%) [4–6]. In contrast, Ferenci *et al.* [24] examined the efficacy of standard-dose Peg-IFN alpha-2a (180 μg weekly) with low-dose ribavirin (400 mg daily) in comparison with standard-dose Peg-IFN alpha-2a (180 μg weekly) and ribavirin (800 mg daily) for genotype 2 and 3 patients, and demonstrated that there was no difference between the two treatment groups with respect to SVR rates (64% with 400 mg/day compared with 69% with 800 mg/day) and relapse rates (20% with 400 mg/day compared

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with 17% with 800 mg/day). These studies showed that either drug dose can be reduced for genotype 2 and 3 patients without compromising antiviral efficacy. In the present study, neither Peg-IFN nor ribavirin drug exposure participated in reaching RVR and SVR. In particular, more than 90% of patients having RVR achieved SVR regardless of the drug exposure level, as long as the mean Peg-IFN dose was over 0.5 μg/kg/week and ribavirin was over 5.0 mg/kg/day. The results of our study suggested that genotype 2 patients may receive reduced levels of both drug doses on the condition that they can complete the full 24-week course of combination therapy. Randomized, prospective trials that reduced both Peg-IFN and ribavirin should be conducted for CHC patients to clarify this.

In the present study, while the treatment outcome was independent of the individual ribavirin exposure in patients who had completed the 24-week treatment, the most common reason to withdraw the treatment was decreased haemoglobin because of ribavirin medication. Based on the results of randomized controlled trials [6], using a ribavirin dose of 800 mg/day is recommended for genotype 2/3 patients [1–3]. However, several studies have shown that some patients cannot tolerate even this suboptimal ribavirin dose. This is a serious problem for patients with the risk of anaemia, especially elderly patients. The ageing of patients is progressing around the world, requiring improvement in treatment tolerability. Recently, Andriulli *et al.* [25] examined the effect of ribavirin in a 12-week course of therapy on CHC genotype 2 patients with RVR in two groups, one continuing with ribavirin and the other receiving Peg-IFN alpha-2a alone after week 6. The relapse rates were higher (46% *vs* 17%; $P < 0.001$) and overall SVR rates were lower (54 *vs* 82%; $P < 0.001$) in patients who stopped receiving ribavirin at week 6. Thus, ribavirin medication throughout the treatment period is necessary to raise the SVR rate even in genotype 2 or 3 patients with RVR. In the present study, the ribavirin dose could be reduced without loss of efficacy for genotype 2 patients, as long as the patients were treated for 24 weeks. Therefore, in the patients with the risk of anaemia, it would be better to reduce the dose of ribavirin before anaemia arises rather than being forced to discontinue the combination therapy because of anaemia caused by ribavirin medication. We previously reported that in CHC patients treated by IFN or Peg-IFN in ribavirin combination therapy, a decline of haemoglobin concentration by 2 g/dL at the end of 2 weeks from the start of the treatment can be used to identify patients likely to develop severe anaemia [26,27]. This kind of predictive factor for the progression to severe anaemia can be of much help in reducing ribavirin with appropriate timing.

Our study has some limitations. First, it is a retrospective study, and we could not obtain complete information for all patients. However, this is the first study of Peg-IFN and ribavirin combination therapy in which the drug dose of Peg-IFN and ribavirin taken by each patient was assessed independently for HCV genotype 2 patients. Our results can be taken as an evidence offering suggestions for the treatment of CHC genotype 2 patients. Second, this cohort included patients with different histories of past IFN treatment. Patients who had failed to recover with previous IFN-based treatment were likely to experience treatment failure again [28]. Therefore, we examined the predictors of treatment response separately according to treatment history, and confirmed that in both naïve and treatment-experienced patients, the mean dose of Peg-IFN and ribavirin showed no correlation with SVR or RVR in both groups.

In conclusion, our study demonstrates that RVR is an important treatment predictor and more than 90% of patients having RVR achieve SVR with combination therapy of Peg-IFN and ribavirin for genotype 2 infected CHC patients regardless of the drug exposure. Further prospective, randomized studies are necessary to assess whether the standard or a reduced dose of each drug can produce equivalent outcomes.

ACKNOWLEDGEMENTS

Other institutions and participants in the Osaka Liver Forum are: Hyogo Prefectural Nishinomiya Hospital, Y. Inui; Osaka Medical Center for Cancer and Cardiovascular Diseases, K. Katayama, K. Imanaka; National Hospital Organization Osaka Minami Medical Center, T. Hijioka; Sumitomo Hospital, A. Yamada; Osaka Koseinenkin Hospital, T. Ito; Suita Municipal Hospital, T. Nagase; Itami City Hospital, T. Kashihara; Otemae Hospital, Y. Doi; Ashiya Municipal Hospital, K. Kiriyama; NTT West Osaka Hospital, A. Kaneko; Osaka Kaisei Hospital, N. Imaizumi; Nishinomiya Municipal Central Hospital, H. Ogawa; Kano General Hospital, S. Kubota; and Saiseikai Senri Hospital, K. Suzuki. This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from Ministry of Health Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science and Culture of Japan.

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Journal of Viral Hepatitis, 2009, 16, 586–594 doi:10.1111/j.1365-2893.2009.01106.x

Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin

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Received November 2008; accepted for publication December 2008

SUMMARY. The impact of ribavirin exposure on virologic relapse remains controversial in combination therapy with pegylated interferon (Peg-IFN) and ribavirin for patients with chronic hepatitis C (CH-C) genotype 1. The present study was conducted to investigate this. Nine hundred and eighty-four patients with CH-C genotype 1 were enrolled. The drug exposure of each medication was calculated by averaging the dose actually taken. For the 472 patients who were HCV RNA negative at week 24 and week 48, multivariate logistic regression analysis showed that the degree of fibrosis ($P = 0.002$), the timing of HCV RNA negativiation ($P < 0.001$) and the mean doses of ribavirin ($P < 0.001$) were significantly associated with relapse, but those of Peg-IFN were not. Stepwise reduction of the ribavirin dose was associated with a stepwise increase in relapse rate from 11% to 60%. For patients with complete early virologic response (c-EVR) defined as HCV RNA negativity at week 12, only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin and ribavirin exposure affected the relapse even after treatment week 12, while Peg-IFN could be reduced to 0.6 μg/kg/week after week 12 without the increase of relapse rate. Ribavirin showed dose-dependent correlation with the relapse. Maintaining as high a ribavirin dose as possible (≥12 mg/kg/day) during the full treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to Peg-IFN alpha-2b plus ribavirin, especially in c-EVR patients.

Keywords: chronic hepatitis C, drug exposure, pegylated interferon plus ribavirin, virologic relapse.

INTRODUCTION

Combination therapy of pegylated interferon (Peg-IFN) plus ribavirin is very effective for patients with chronic hepatitis C (CH-C). However, sustained virologic response (SVR) in current therapy occurs in only 40–50% of patients with hepatitis C virus (HCV) genotype 1 [1–4]. Also, SVR is reduced in patients with genotype 1 who require reduction of either Peg-IFN or ribavirin, although dose reduction has little influence on SVR in those with genotype 2 or 3 [1–3,5,6]. Therefore, it is important to clarify the degree to which these medications can be reduced without adversely affecting SVR in patients with CH-C genotype 1.

In an early report on the relationship between drug exposure and antiviral effect in patients with CH-C genotype 1, patients who received ≥80% of their total planned cumulative doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy had an SVR of 51% compared with only 34% for patients who received lesser amounts of one or both

Abbreviations: CH-C, chronic hepatitis C; c-EVR, complete early virologic response; ETR, end-of-treatment virologic response; Hb, haemoglobin; HCV, hepatitis C virus; IFN, interferon; LVR, late virologic response; Peg-IFN, pegylated interferon; PP, per protocol; Plt, platelet; RVR, rapid virologic response; SVR, sustained virologic response; VR, virologic response; WBC, white blood cell.

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medications [7]. On the other hand, Shiffman *et al.* [8] recently reported that reducing ribavirin did not affect SVR as long as the dose of Peg-IFN was maintained, while reducing the Peg-IFN dose significantly reduced SVR. The results of these observations are consistent with respect to the effect of Peg-IFN on SVR. However, what is controversial is whether or not reducing the ribavirin dose affects the antiviral effect.

Adding ribavirin to either interferon (IFN) or Peg-IFN monotherapy for patients with CH-C genotype 1 has been shown to reduce the relapse rate in large randomized trials [1,2,9–11]. In detail, adding ribavirin to the usual IFN monotherapy (3MIU, three-times-weekly) in 48-week treatment raised the end-of-treatment virologic response (ETR) rate from approximately 30% to 50% and also lowered the relapse rate from mid-40% to approximately 20% [9–11]. Lindsay *et al.* [12] reported that Peg-IFN alpha-2b (Peg-IFN α-2b) monotherapy (1.5 μg/kg, once-weekly), as compared with IFN alpha-2b (IFNα-2b) monotherapy (3MIU, three-times-weekly), improved ETR (49% vs. 24%), but not the relapse rate (53% vs. 50%). In the trial of Peg-IFN alpha-2a (Peg-IFN α-2a) plus ribavirin *vs* IFN α-2b plus ribavirin or Peg-IFN α-2a alone, the ETR rates were 69%, 52% and 59%, and the relapse rates were 19%, 15% and 52%, respectively [2]. These findings from large-scale trials indicate that the main role of ribavirin is to reduce relapse in the combination therapy with Peg-IFN, although ribavirin affects both ETR and relapse in combination therapy with the usual IFN.

In the present study, we tried to determine whether or not dose reduction of ribavirin (or Peg-IFN) has an effect on virologic relapse in Peg-IFN plus ribavirin treatment for patients with CH-C genotype 1.

PATIENTS AND METHODS

Patients

This study was a multicentre trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C were enrolled in this study between December 2004 and September 2006, and treated with a combination of Peg-IFN α-2b plus ribavirin. The baseline characteristics of the patients are shown in Table 1. All patients were Japanese infected with HCV genotype 1 and a viral load of more than 10^5 IU/mL. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Treatment

All patients received Peg-IFN α-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough) for the duration of the study of 48 weeks. As a starting dose, Peg-IFN α-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg) according to the manufacturer's drug information available in Japan.

Table 1 Baseline characteristics of patients and drug doses at start of treatment

Factor	Mean ± SD or *n*
n	984
Age (years)	56.3 ± 10.1
Sex (male/female)	555/429
Body weight (kg)	61.8 ± 11.5
History of IFN treatment Naïve/experienced (relapser/nonresponder)*	575/409 (160/182)
White blood cells (/mm^3)	5052 ± 1550
Neutrophils (/mm^3)	2577 ± 1092
Red blood cells (×10^4/mm^3)	442 ± 47
Haemoglobin (g/dL)	14.1 ± 1.4
Platelets (×10^4/mm^3)	15.9 ± 5.5
AST (IU/L)	66 ± 45
ALT (IU/L)	79 ± 61
Serum HCV RNA (kIU/mL)†	1600
Histology (METAVIR)‡	
Fibrosis; 0/1/2/3/4	49/314/197/105/18
Activity; 0/1/2/3	23/329/304/27
Peg-IFN dose (μg/kg/week)	1.45 ± 0.17
Ribavirin dose (mg/kg/day)	11.4 ± 1.6

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus. *Viral response to previous treatment was unknown in 57 patients, and 10 patients had discontinued treatment. †Data shown are median values. ‡301 missing.

Dose reduction and discontinuance

Dose modification also followed, as a rule, the manufacturer's drug information according to the intensity of the haematologic adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below 1500/mm^3, the neutrophil count below 750/mm^3 or the platelet (Plt) count below 8×10^4/mm^3, and was discontinued when the WBC count was below 1000/mm^3, the neutrophil count below 500/mm^3 or the Plt count below 5×10^4/mm^3. Ribavirin was also reduced from 1000 mg to 600 mg, 800 mg to 600 mg, or 600 mg to 400 mg when the haemoglobin (Hb)

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concentration decreased to less than 10 g/dL, and was discontinued when the Hb concentration decreased to less than 8.5 g/dL. Both Peg-IFN α-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. No ferric medicine or haematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor, were administered.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics). Complete early virologic response (c-EVR) was defined as the absence of detectable serum HCV RNA at treatment week 12, the late virologic response (LVR) was defined as undetectable serum HCV RNA for the first time at 13–24 weeks of treatment, and the virologic response (VR) was defined as HCV RNA negativity at week 24 and week 48. SVR was defined as the absence of detectable serum HCV RNA at week 72. Patients with less than a 2-log decrease in HCV RNA level at treatment week 12 compared with the baseline had to stop treatment according to the protocol and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered to be nonresponders and were excluded from further treatment.

Assessment of drug exposure

The amounts of Peg-IFN α-2b and ribavirin actually taken by each patient during the full treatment period were evaluated by reviewing the medical records. The mean doses of Peg-IFN α-2b and ribavirin were calculated individually as averages on the basis of body weight at baseline: Peg-IFN α-2b expressed as μg/kg/week, ribavirin expressed as mg/kg/day.

Evaluation of impact of drug exposure on virologic relapse

We evaluated the relationship between the drug exposure of both drugs and relapse by two different methods, univariate and multivariate analysis for relapse and independent evaluation of both drugs for relapse according to the degree of drug exposure. The former was performed with the factors of mean administration doses of both drugs, including the factors at baseline and the timing of HCV RNA negativiation. The latter was examined by classifying Peg-IFN α-2b exposure into five categories (up to 0.6 μg/kg; from 0.6 to less than 0.9 μg/kg; from 0.9 to less than 1.2 μg/kg; from 1.2 to less than 1.5 μg/kg; from 1.5 μg/kg) and ribavirin exposure into five categories (up to 6 mg/kg; from 6 to less than 8 mg/kg; from 8 to less than 10 mg/kg; from 10 to less than 12 mg/kg; from 12 mg/kg).

Statistical analysis

Baseline data are expressed as means ± SD or median values. Virologic response was evaluated using per protocol (PP) analysis. To analyse the difference between baseline data including drug exposure and virologic response, univariate analysis using the Mann–Whitney *U*-test or chi-square test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. A two-tailed *P* value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

RESULTS

Progress of patients and dose reduction of Peg-IFN α-2b and ribavirin

The progress of patients in this study is shown in Fig. 1. Of the 984 patients, 903 completed 12 weeks of treatment and the c-EVR rate was 49% (445/903), based on PP study. To analyse for relapse, 472 patients with VR were assessed, with 178 (38%) showing Peg-IFN dose reduction without discontinuation and 246 (52%) with ribavirin dose reduction without discontinuation during the full (48 weeks) treatment period. The relapse rate was 26% (125/472) in the patients with undetectable HCV RNA level at the end of treatment. No difference was found in relapse rates between the IFN naïve patients and IFN experienced patients (IFN naïve; 25%, 72/287 *vs* IFN experienced; 29%, 53/185, *P* = 0.40). The SVR rate was 43% (347/812) in the PP study.

Impact of drug exposure during 0–48 weeks on relapse among patients with VR

The mean dose of Peg-IFN α-2b actually taken during the full treatment period by each patient was 1.32 μg/kg/week (range, 0.49–2.16 μg/kg/week; median, 1.38 μg/kg/week) and that of ribavirin was 9.8 mg/kg/day (range, 3.3–16.2 mg/kg/day; median, 10.1 mg/kg/day) in patients with VR.

The result of univariate analysis for relapse among the patients with VR is shown in Table 2a. The degree of fibrosis, the timing of HCV RNA negativiation, Plt value and the mean doses of ribavirin were factors significantly associated with relapse, but those of Peg-IFN α-2b were not. The mean dose of ribavirin as well as the degree of fibrosis and the timing of HCV RNA negativiation was selected as a significant independent factor by multivariate logistic regression analysis (Table 2b).

Next, we analysed the relationship of the relapse rate and the mean ribavirin dose. The overall relapse rate among patients with VR was 26% (125/472). The

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