Discussion

When using PTA in patients with HCC, the tumor must be clearly visualized on US to avoid complications and to treat the tumor successfully.[12] Recently, percutaneous RFA has become widely used in HCC patients owing to its safety and efficacy.[1-3] However, RFA is difficult when the tumor is located under the hepatic dome or near the surface of the liver, since the tumor is then poorly visible on US, or adjacent structures, such as the diaphragm or skin, can be burned. Several approaches, such as PTA assisted by thoracoscopy[4] or laparoscopy[5] under general anesthesia, can be used to overcome these limitations and obtain good results. As well, the use of noninvasive methods, such as artificial pleural effusion[7-9] and artificial ascites,[10-11] has been reported. However, previously these methods have been used separately. In this study, the safety and local efficacy of RFA with both artificial pleural effusion and ascites was evaluated.

When performing PTA, it is also important that the needle tract be visualized clearly on US without any structures, such as the hepatic or portal vein, intervening. We induced artificial pleural effusion when the tumor or the needle tract could not be visualized clearly on US because the tumor was located under the hepatic dome or the hepatic vein blocked the view. Artificial ascites was induced in lesions in which the tumor or the needle tract was not clearly visible on US after artificial pleural effusion was induced; artificial ascites was also induced when the tumor was located near the surface of the liver to avoid thermal injury to adjacent structures. Using these procedures, we succeeded in overcoming some of the limitations of percutaneous, US-guided RFA, and the treatment was performed safely in all patients. However, in patients with a past history of a pulmonary or abdominal operation, which can cause intra-abdominal adhesions, the use of this method may be limited, although we did not experience any such difficulties,

In this study, the combination of artificial pleural effusion and artificial ascites was used in patients having lesions under the hepatic dome and near the surface of the liver. If the tumor had been ablated without inducing artificial ascites in these patients, all of the tumor might not have been ablated owing to pain. Adhesions between the liver and the diaphragm might also have occurred as a result of a diaphragm burn. Of note, intra-abdominal adhesions limit the ability to successfully treat HCC patients noninvasively. Indeed, most patients with artificial ascites showed decreased pain during ablation. All but one patient could be ablated completely without severe pain. The induction of artificial ascites did not cause ablation to be discontinued in any patients. Since HCC patients can be expected to have recurrences, the initial choice of method should take into account the treatment of the next recurrence. Koda et al.[7] reported that percutaneous RFA with artificial pleural effusion was useful, and, although they did not mention the possible complication of adhesions developing between the liver and diaphragm, they did report difficulty in inducing artificial ascites in the subphrenic space. To overcome this difficulty, we began ablating the tumor as soon as the space between the liver and the diaphragm became visible on US, before the fluid could escape into the abdominal cavity. At present, RFA with the combination of artificial pleural effusion and artificial ascites is still not an established method. However, we identified advantages of performing RFA with this combination instead of a artificial pleural effusion or artificial ascites, and we were able to perform the treatment safely.

Complications due to RFA done with artificial pleural effusion and/or artificial ascites were seen in 3 of 43 HCC lesions (7.0%). Pneumonia occurred in one patient and atelectasis in another; both resolved within a week. A liver abscess was observed in one patient and was treated successfully with antibiotics and drainage. The pneumonia and atelectasis were likely secondary to the artificial pleural effusion, and the liver abscess might have been a result of the RFA. These patients with complications were among the first we treated using this approach; more recently, we have found no such complications. In contrast, with conventional RFA, 21 (8.0%) of 263 treated HCC lesions were associated with complications.[13-17] Thoracic complications were seen in six patients (thoracic cavity bleeding in one, pleural effusion in three, atelectasis in two), abdominal bleeding in four, liver infarction in three, and a biloma developed in eight patients. The mortality rate related to conventional RFA has been reported to range from 0.2% to 0.7%;[18] however, there have been no deaths with our method. Owing to the lack of a prospective control group treated with conventional RFA, we cannot precisely compare the frequency of these complications. Nevertheless, the complications with artificial pleural effusion and/or artificial ascites were not serious and resolved easily.

Although the definition of "safety margins" for local efficacy is controversial in the treatment of HCC, it is important to ablate enough tissue to achieve complete necrosis and thus prevent local recurrence. To achieve this, artificial ascites is useful for creating a space between the liver and adjacent structures, which allows adequate safety margins as determined by CT, without any complications. Accumulation of lipiodol on CT is useful for judging the local efficacy of RFA, since one can distinguish the lipiodol accumulation area from the surrounding treatment area. In this study, all lesions were evaluated as having received adequate ablation by

comparing postablation CT images with CT images obtained before treatment. However, when lipiodol is not injected, it is difficult to tell whether the ablated area completely surrounds the original tumor. In one case, lipiodol was not injected owing to obstruction by the common hepatic artery. In this study, there were no cases of local recurrence (0%).[19-20] The injection of lipiodol into the feeding artery might play an important role in evaluating treatment efficacy in a hypervascular tumor. Although tumor seeding has been reported after PTA,[18] seeding in the pleural cavity or abdominal cavity was not observed in this study.

We previously reported that virtual US was useful in dealing with tumors that were seen faintly on US with the combination of artificial pleural effusion and artificial ascites.[21]

During the observation period of 31.8 ± 5.8 months, two patients died of recurrent HCC and liver failure. The aim of this study was not to evaluate long-term survival; thus, a study evaluating long-term survival with the use of this method needs to be done in the near future.

In conclusion, percutaneous RFA with artificial pleural effusion and/or artificial ascites appears to be a safe and effective treatment for obtaining good local control of HCC.

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Hepatitis C Virus Expression and Interferon Antiviral Action Is Dependent on PKR Expression

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Interferon (IFN)-inducible double-stranded RNA-activated protein kinase (PKR) is thought to play a key antiviral role against hepatitis C virus (HCV). However, demonstrating the importance of PKR expression on HCV protein synthesis in the presence or absence of IFN has proven difficult in vivo. In the present experiment, full-length HCV constructs were transiently transfected into two cell lines stably expressing T7 RNA polymerase. HCV expression was monitored under conditions of upregulated or downregulated PKR expression. In addition, IFN was monitored during downregulation of PKR. HCV expression effectively increased PKR expression, as well as that of its regulated proteins. PKR was obviously knocked down by PKR-specific siRNA, which resulted in significantly increased HCV core protein levels. Conversely, over-expression of PKR significantly suppressed HCV core levels in both cell lines. Furthermore, IFN induced high levels of PKR, whereas downregulation of PKR reversed IFN's antiviral effects and increased HCV core levels. Based on these results, it appears that HCV protein expression is directly dependent on PKR expression. PKR is antiviral toward HCV and responsible for IFN's effect against HCV. ***J. Med. Virol. 79:1120–1127, 2007.***

KEY WORDS: small interfering RNA (siRNA); eukaryotic initiation factor-2α (eIF2α); BT7-H; Huh-T7

INTRODUCTION

Interferon (IFN)-inducible double-stranded RNA-activated protein kinase (PKR) appears to play a key antiviral role against hepatitis C virus (HCV). PKR is one of a number of host IFN-stimulated genes (ISGs) [Sen and Ransohoff, 1993]. Nearly all mammalian cells express PKR at low levels [Kaufman, 2000]. Double-stranded-RNA (dsRNA), produced during RNA viral replication, is a potent activator of PKR [Meurs et al., 1993]. Activated PKR in turn induces phosphorylation of PKR and eukaryotic initiation factor-2α (eIF2α), which inhibits protein synthesis, including that of virally encoded proteins [Samuel, 1979]. PKR appears to play multiple roles in cell growth, differentiation, apoptosis, oncogenesis, and responses to cellular stresses, such as infection [Gale et al., 2000]. However, proof-proving inhibition of HCV protein synthesis by PKR is still lacking [Koev et al., 2002; MacQuillan et al., 2002; Vyas et al., 2003]. While HCV structural protein E2 and nonstructural protein NS5A appear to block activation of PKR, it remains unclear whether these proteins contribute to HCV persistence or resistance to IFN in vivo [Francois et al., 2000; Gerotto et al., 2000; Taylor et al., 2001]. Furthermore, it remains controversial whether PKR is required for elimination of HCV in patients treated with IFN [MacQuillan et al., 2003; Giannelli et al., 2004].

To analyze the precise interaction between PKR and HCV proteins, we used a full-length HCV cell-based expression system as described in a previous report [Lin et al., 2005]. This expression system uses a wild-type H77 sequence (genotype 1a), capable of infecting chimpanzees with no adaptive mutations. It can produce HCV negative-strand RNA in host cell lines stably expressing T7 polymerase [Hiasa et al., 2006]. It is suitable to analyze interactions between HCV and PKR since it utilizes full-length wild-type HCV RNA with no

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adaptive mutations. Moreover, it is capable of synthesizing double-stranded HCV-RNA within host cell lines in which particular IFN signaling pathways have been activated. Moreover, cell-based systems are capable of expressing HCV proteins only by plasmid transfection. This expression system results in efficient production of HCV proteins with minimal artificial effects. Using this HCV cell-based expression system, we explored the relationship between PKR and HCV full genome protein expression in two independent cell lines.

Clinically, IFN remains the only drug capable of eliminating HCV. At present, pegylated IFN-α in combination with ribavirin is standard therapy for patients with HCV. Although PKR is thought to play an important role in IFN's control of HCV protein expression, a number of independent studies suggest that HCV can be suppressed by IFN by mechanisms other than activation of PKR [Francois et al., 2000; Guo et al., 2003, 2004]. Thus, it is still not clear whether PKR is solely responsible for IFN's antiviral effects against HCV. In the present study, first we examined the impact of PKR on HCV protein expression using our binary plasmid-based HCV expression system. We then investigated the role of PKR in mediating IFN's antiviral effects against HCV.

MATERIALS AND METHODS

Cells

Huh-T7 cells were generated by stably transfecting bacteriophage T7 RNA polymerase into Huh-7 cells [Schultz et al., 1996], and BT7-H cells were generated by stably transfecting T7 into African green monkey kidney cell lines BS-C-1 [Whetter et al., 1994] (both gifts of Dr. Stanley M. Lemon, University of Texas, Galveston). Both cell lines were grown in Dulbecco's modified Eagle's medium (D-MEM) (Gibco-BRL, Gaithersburg, MD). For BT7-H cells, we added 500 μg/ml gentamicin sulfate, and for Huh-T7 cells, we added 250 μg/ml gentamicin sulfate to the culture medium.

Plasmid and Cell Transfection

pH77 is a full-length HCV genotype 1a construct [Chung et al., 2001]. Briefly, a plasmid containing a full-length genotype 1a cDNA sequence corresponding to the H77 prototype strain [Yanagi et al., 1997] was adapted at its 5′ and 3′ termini with a T7 promoter and a hepatitis delta virus ribozyme sequence, respectively, to yield pT7-flHCV-Rz (hereafter referred to as pH77). Plasmid pcDNA-PKRwt (pPKR) expressing wild-type PKR was a kind gift from Dr. Michael Gale, Jr. (University of Texas Southwestern) [Meurs et al., 1993]. In order to induce over-expression of PKR, we co-transfected pPKR and pH77 into cells. The plasmid pOS8, expressing β-galactosidase under control of the T7 promoter, was used as control plasmid [Chung et al., 2001]. Transfection of each plasmid was performed using Lipofectamine Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. In a 6-well tissue culture plate, cells were seeded in 2 ml of medium. For each transfection, 3 μg of plasmid DNA was used. The transfected cells were harvested at different time points.

Synthesis and Transfection of PKR-Specific siRNA

We designed a PKR-specific siRNA (PKRsi-1: GAA CUG CCU AAU UCA GGA C, nt. 520–540) from a PKR sequence template (accession number NM002759) purchased from Dharmacon Research, Inc. (Lafayette, CO). To design PKR-specific siRNA, the mRNA sequence of PKR was screened using the National Center for Biotechnology Information database and the BLAST search algorithm. Cy3 labeled luciferase GL2 duplex (Dharmacon) was used as a control siRNA (Control-si). In order to induce downregulation of PKR, we transfected siRNA 48 hr prior to transfection with pH77. Transfection with 50 pmol of siRNA was performed using siFECTOR (B-bridge International, Sunnyvale, CA). pH77 was transfected using Lipofectamine reagent (Invitrogen).

Cell Culture With or Without IFN

For assays using IFN, cells were cultured in the presence of 100 IU/ml IFNα-2b (Schering-Plough, Kenilworth, NJ) 4 hr after transfection. Medium with or without IFN was changed at Day 1 post-infection and every 2 days thereafter.

Western Blotting

Cells were washed twice with phosphate buffered saline (PBS) and lysed with 100 μl of RIPA buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM of NaCl, 1% sodium dodecyl sulfate). Thirty micrograms of lysate protein were used, and separated by electrophoresis on 4–12% Bis–Tris gradient gel (Invitrogen), then blotted onto Immobilon-P membranes (Millipore, Bedford, MA). Each membrane was then incubated with the relevant antibody. An ECL plus kit (Amersham Pharmacia, Buckinghamshire, UK) was used for detection. Monoclonal antibody to human PKR and polyclonal antibody to eIF2α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while polyclonal antibody to phosphorylated PKR was obtained from BioSource (Camarillo, CA), and polyclonal antibody to phosphorylated eIF2α peptide was obtained from ResGen (Invitrogen). Monoclonal antibody to actin was obtained from Chemicon International (Temecula, CA). Appropriate species-specific conjugated secondary antibodies were obtained from commercial kits (Amersham Pharmacia). Autoradiograms were scanned, and the signal intensity of each band analyzed using Scion Image software (Scion Corporation, Frederick, MD).

ELISA Assay for HCV Core Antigen

Cell culture lysates were adjusted to 20 μg/ml. HCV core antigen concentrations were quantified using the

HCV core antigen ELISA test (Ortho-Clinical Diagnostics, Osaka, Japan) following the manufacturer's instructions. HCV core ELISA data were expressed in terms of fmol of HCV core antigen per 20 μg of total protein.

Cellular RNA Extraction and Real-Time RT-PCR for PKR and GAPDH mRNA

Total RNA was extracted with TRIzol reagent (Invitrogen) and treated two times for 4 hr with DNase I using a DNase-free kit (Ambion, Austin, TX). RNA was adjusted to 0.3 μg/μl. Reverse transcription was performed as described previously [Hiasa et al., 2003]. PKR and GAPDH cDNA were then quantified by real-time PCR using LightCycler technology and SYBR green I dye (Roche Diagnostics, Mannheim, Germany). Real-time PCR for PKR was performed with 2 μl of purified cDNA in a reaction SYBR green I mixture containing 4 mM $MgCl_2$, and 5 pM each of forward primer (5′-AGCACACTCGCTTCTGAATC-3′) and reverse primer (5′-CTGGTCTCAGGATCATAATC-3′) [Hiasa et al., 2003]. The PCR consisted of an initial denaturation step for 10 min at 95°C, then 40 cycles under the following conditions: 10 sec at 95°C, 10 sec at 58°C, and 15 sec at 72°C. For PCR amplification of GAPDH, we used a commercial GAPDH primer set (Roche Search LC, Mannheim, Germany), with conditions as recommended by the manufacturer. For analysis of PKR mRNA, we determined PKR mRNA copy numbers, which were normalized to the GAPDH copy number, to provide standardized values.

Statistical Analysis

Each analysis was performed in at least quadruplicate to arrive at a mean value and SE. Data were analyzed statistically using SPSS 10.0J software (SPSS, Chicago, IL). Mean value differences were analyzed by the Mann–Whitney *U* test. *P*-values < 0.05 were considered to be significant.

RESULTS

Expression of HCV Activates PKR in This Cell-Based System

We first examined the level of HCV core protein expression in our cell-based system, and monitored the time course of HCV core protein expression in BT7-H and Huh-T7 cell lines stably expressing T7 polymerase. We have previously demonstrated that transfection of pH77 (containing full-length HCV cDNA of genotype 1a) leads to successful HCV replication in these cell lines [Hiasa et al., 2006]. The basal level of HCV core protein expression in BT7-H cells was approximately 10 times greater than in Huh-T7 cells, however, expression quickly fell by Day 7 (Fig. 1A). As determined by Western blot analysis, an increase in phosphorylated PKR with HCV expression was observed, and increased amounts of PKR and phosphorylated eIF2α (indicative of PKR activity) were also observed from Days 2 to 5 in BT7-H cells (Fig. 1C). In Huh-T7 cells, less significant increases in PKR protein, as well as phosphorylated PKR and phosphorylated eIF2α, were observed than in BT7-H cells. A slight increase in phosphorylated eIF2α was observed on Day 5. The initial peak in HCV protein expression in BT7-H cells appeared to activate PKR by phosphorylation. In contrast, transfection with pOS8 did not increase phosphorylated PKR and eIF2α levels in either cell line. These results suggest that HCV actually enhances PKR function in this model. These results led us to investigate the effect of PKR over-expression on HCV expression.

Over-Expression of PKR Suppresses HCV Core Protein Expression

To determine whether PKR inhibits HCV protein expression, we over-expressed PKR in cell lines using a PKR expression plasmid (pPKR) [Pflugheber et al., 2002]. We co-transfected pH77 with either pPKR or pOS8 (a control plasmid expressing β-galactosidase). As demonstrated by Western blot analysis, pPKR efficiently expressed both PKR and phosphorylated PKR protein in excess of control levels in BT7-H cells (Fig. 2A). Strong expression of PKR continued until Day 5. pPKR also strongly expressed PKR protein in Huh-T7 cells (data not shown). Over-expression of PKR was capable of inhibiting HCV core protein in both cell lines (Fig. 2B). PKR significantly inhibited HCV core protein expression in BT7-H cells from Days 1 to 5 ($P < 0.05$), and in Huh-T7 cells from Days 2 to 4 ($P < 0.05$). These results suggest that PKR can efficiently inhibit HCV protein expression and overcome functional inhibition by HCV proteins, such as E2 and NS5A.

Maintenance of HCV Core Protein Expression Occurs in Cells With Downregulated PKR

We next explored the time course of HCV core protein expression with regard to downregulation of PKR expression. We sought to determine whether HCV replication might be sustained by inhibiting PKR. To silence PKR expression, we designed siRNA targeting the open reading frame of PKR (PKRsi-1), and evaluated the extent of PKR downregulation in both cell lines (Fig. 3A). Transfection with PKRsi-1 resulted in downregulation of PKR mRNA for 7 days (Fig. 3A). When HCV plasmid (pH77) was introduced after downregulation of PKR, the overall increase in subsequent expression of PKR was partially decreased by PKR siRNA (Fig. 3B).

After confirming downregulation of PKR by siRNA, we evaluated the effect of PKR siRNA on HCV protein expression in cell culture to determine whether PKR influences HCV replication. Compared with control siRNA (Control-si), PKRsi-1 significantly increased HCV expression in both cell lines from Days 3 to 5 (Fig. 4). The level of upregulation of HCV core expression was significant in both cell lines (PKRsi-1: $3{,}770.1 \pm 442.0$ vs. Control-si: $2{,}057.1 \pm 329.5$ fmol/

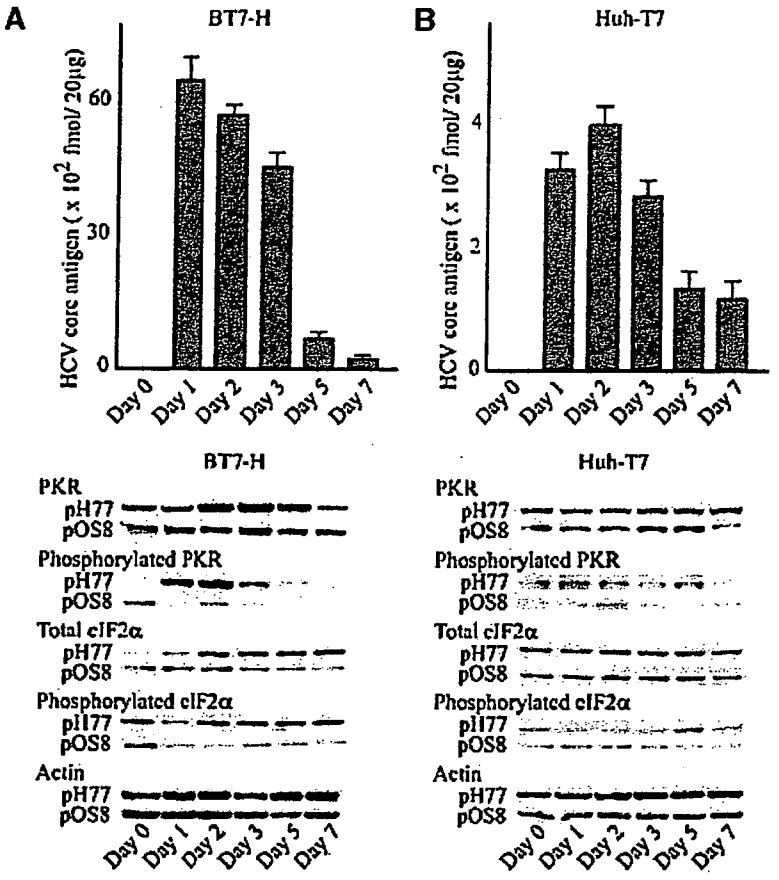

Fig. 1. HCV core protein was efficiently expressed using the binary replication system. HCV expression induced and activated PKR. **A:** A plasmid containing full-genome HCV cDNA (pH77) was transfected into BT7-H and Huh-T7 cell lines. Each line was stably transfected with a T7 polymerase gene. Following transfection of pH77, HCV core protein was expressed for at least 7 days. Results are expressed as the means ± SE of six experiments. **B:** The plasmid pH77 or pOS8 was transfected into BT7-H and Huh-T7 cells. Protein levels of PKR, phosphorylated PKR, total eIF2α, phosphorylated eIF2α, and actin were analyzed by Western blot. In BT7-H cells, PKR was stimulated and activated by HCV expression. In Huh-T7 cells, PKR was partially activated, however, activation was minimal compared to BT7-H cells.

20 μg, on Day 4 in BT7-H cells ($P < 0.01$); PKRsi-1: 448.2 ± 126.4 vs. Control-si: 274.0 ± 14.2 fmol/20 μg, on Day 4 in Huh-T7 cells ($P < 0.01$)) (Fig. 4). These results indicate that PKR directly influences on HCV replication in these cell systems.

Interferon's Anti-HCV Effects are Dependent on PKR Expression

IFN induces PKR expression, however, it is still controversial whether PKR is required for elimination of HCV in infected patients treated with IFN [MacQuillan et al., 2003; Giannelli et al., 2004]. Based on our observations, over-expression of PKR inhibits HCV protein expression. To determine whether PKR is essential to IFN's antiviral activity, the effect of IFN-α on HCV-infected cells with downregulated PKR was examined. We transfected PKRsi-1 into each cell line to inhibit the expression of PKR. After 48 hr, we transfected pH77 to induce expression of HCV protein, then added 100 IU/ml of IFN to the culture medium of selected wells. The cells were harvested from Days 1 to 5 following pH77 transfection. IFN stimulated PKR expression in both cell lines, and strong expression of PKR was observed in cells transfected with control siRNA (Control-si) by Western blot (Fig. 5A). As expected, the cells treated with IFN demonstrated reduced HCV core protein levels compared with IFN untreated, control si-transfected cells. On the other hand, in cells transfected with PKRsi-1, PKR expression was markedly reduced in the presence of IFN, especially prior to Day 3 (Fig. 5A). In cells transfected with control-si, HCV core protein levels were reduced by IFN (Fig. 5B). In contrast, in cells with PKR downregulation, significant increases in HCV core protein expression were observed compared with Control-si transfection at each time point in both cell lines ($P < 0.05$). These data confirm that PKR is an important mediator of IFN's antiviral effects against HCV.

DISCUSSION

In the present study, we used a full-length HCV cell-based expression system to demonstrate that PKR influences HCV expression. Sufficient HCV protein expression for at least 7 days was achieved using this

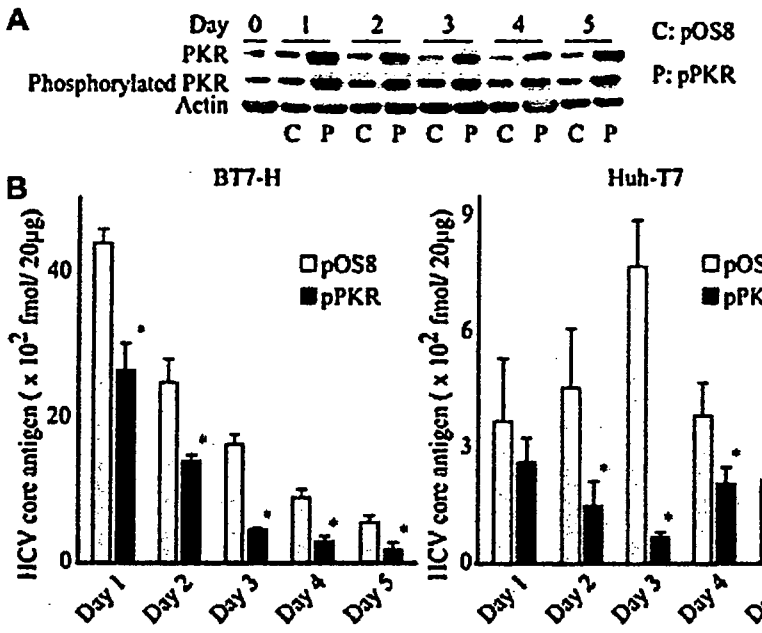

Fig. 2. Over-expression of PKR inhibited HCV core expression. A: pH77 and a PKR expression vector (pPKR) were co-transfected into BT7-H cells. A plasmid expressing β-galactosidase (pOS8) under the T7 promoter was used as a control. pPKR markedly upregulated PKR and phosphorylated PKR expression. B: After over-expression of PKR, significant inhibition of HCV core protein expression was observed. Results are expressed as the means ± SE of four experiments. $^*P < 0.05$ compared with control. C, pOS8; P, pPKR.

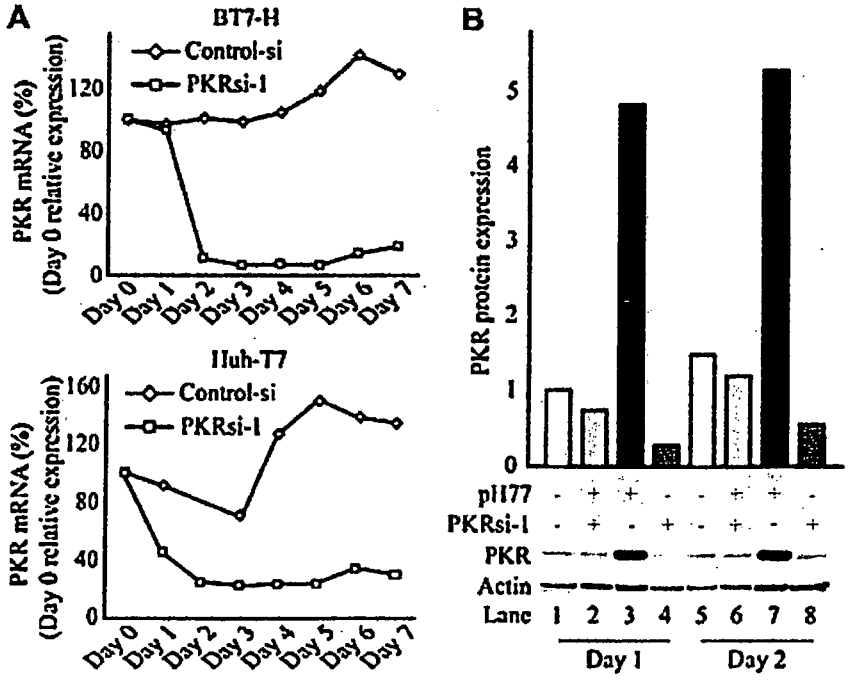

Fig. 3. PKR-specific siRNA efficiently downregulated PKR. A: When BT7-H and Huh-T7 cells were transfected with PKR-specific siRNA (PKRsi-1), marked downregulation of PKR mRNA was observed, compared with use of control siRNA (Control-si). This downregulation of PKR mRNA continued until Day 7. PKR mRNA was expressed as a ratio of GAPDH mRNA. Each value shown indicates relative expression from Day 0. Results are expressed as the mean of two experiments. B: Transfection of BT7-H cells with PKRsi-1 was followed 48 hr later by pH77 transfection. Expression of PKR was evaluated by Western blot, then analyzed by Scion image software, and plotted as a bar graph. pOS8 was used as a control plasmid for pH77, and Control-si was used as a control for PKRsi-1 (both are indicated by (-)). PKRsi-1 downregulated PKR expression effectively (**lane 1** vs. **lane 4**, **lane 5** vs. **lane 8**). In the presence of HCV expression, PKR was stimulated (**lane 1** vs. **lane 3**, **lane 5** vs. **lane 7**). Nevertheless, PKRsi-1 still efficiently inhibited PKR expression, compared to control siRNA (**lane 2**, **lane 6**).

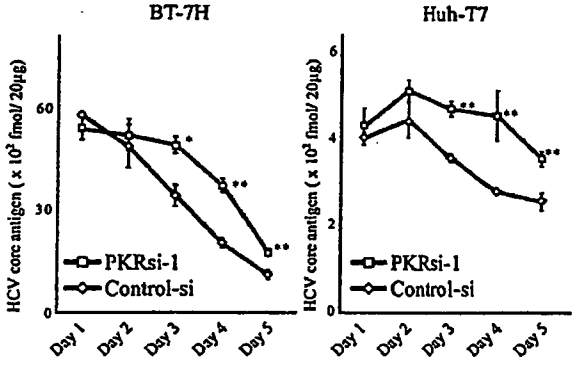

Fig. 4. PKR-silenced cells demonstrated prolonged HCV replication. PKR was downregulated by PKR-specific siRNA (PKRsi-1). Forty-eight hours after transfection with siRNA, we transfected pH77 and evaluated HCV core expression by ELISA assay. The cell lysates were harvested on Days 0–5 after transfection with pH77, and equal quantities of lysate were assayed for HCV core antigen. Data of PKR knockdown BT7-H or Huh-T7 cells using PKRsi-1 were compared with BT7-H or Huh-T7 cells transfected with control siRNA (Control-si). Error bars indicate the means ± SE of five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

expression system by transfection with pH77. HCV replication activated PKR, especially within BT7-H cells. Over-expression of PKR inhibited HCV protein expression, and downregulation of PKR enhanced HCV protein expression. When we added IFN, HCV protein

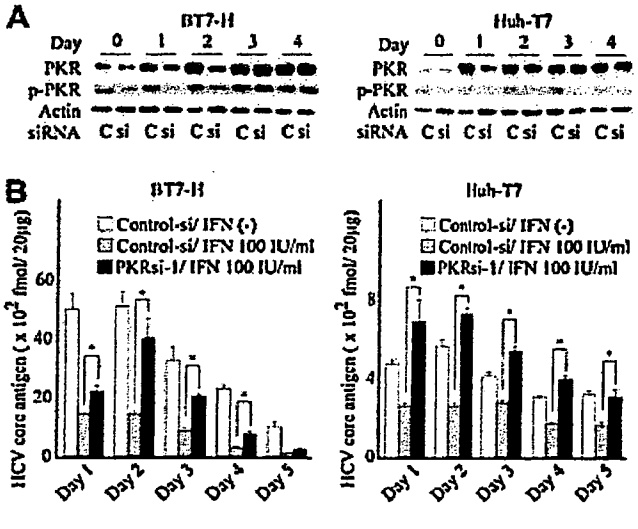

Fig. 5. Inhibition of HCV expression by IFN was PKR-dependent. A: Interferon (IFN) upregulated PKR in pH77 transfected cells. Nevertheless, PKR-specific siRNA (PKRsi-1) efficiently downregulated PKR expression and PKR phosphorylation in both cell lines, especially in the first 3 days. C, Control-si; si, PKRsi-1; p-PKR, phosphorylated PKR. B: Silencing of PKR by PKRsi-1 contributed to a diminished IFN effect on HCV protein expression. Cells were harvested daily, and HCV core protein measured by ELISA assay. Inhibition of HCV core protein by IFN was observed in control-si transfected cells compared with IFN untreated, control siRNA-treated cells. In contrast, despite treatment with IFN, the expression of HCV core protein was significantly increased at all time points in PKRsi-1 transfected cells compared with control-si transfected cells. These results indicate that IFN's antiviral effects against HCV are PKR-dependent. Results are expressed as the means ± SE of four experiments. $^{*}P < 0.05$; PKRsi-1 cells were compared with Control-si cells with IFN.

expression was inhibited in a dose-dependent manner within this system. Downregulation of PKR partially overcame this inhibition by IFN, resulting in increased HCV protein synthesis. These results indicate that HCV expression is directly dependent on PKR, and also indicates that PKR is a key player in IFN's sustained effects against HCV.

Several reports have described a role of PKR in HCV infection, as well as in mediating IFN's antiviral effects. PKR mRNA is significantly over-expressed in the hepatic tissue of patients with chronic hepatitis C, compared with other causes of liver damage [Yu et al., 2000]. However, other reports conclude that PKR protein expression is not upregulated in viral liver disease, including chronic hepatitis C [MacQuillan et al., 2002]. In addition, IFN is the only drug that can eliminate HCV, and it stimulates a number of IFN-stimulated genes, including PKR, 2′5′-oligoadenylate synthetase, and MxA [Sen and Ransohoff, 1993]. Among these, PKR is thought to play an important antiviral role against HCV. Determining the precise role of PKR in HCV infection, as well as the role of PKR in IFN treatment aimed at eliminating HCV, is important to better understand the nature of HCV persistence and to establish a suitable therapeutic protocol for IFN. Thus, we used a recently established full-length HCV cell-based expression system. This system expresses all HCV proteins from wild-type HCV sequences capable of infecting chimpanzees [Lin et al., 2005] without any adaptive mutations. Several reports using HCV replicon models suggest that PKR may mediate viral replication [Pflugheber et al., 2002; Rivas-Estilla et al., 2002; Chang et al., 2006]. However, replicon systems are subgenomic and cannot assess the effects of an entire HCV polyprotein, including E2 and other structural proteins. Since HCV E2 has been reported to impair PKR function [Taylor et al., 1999], and since HCV core protein has also been reported to activate PKR [Theodore and Fried, 2000], a system expressing the full-genome HCV sequence is better suited to analyzing interactions between HCV proteins and PKR. Our system expresses all proteins of the full-length HCV genotype 1a sequence, and therefore incorporates the effects of HCV core and E2 proteins. Recently, an infectious model of HCV genotype 2a capable of producing virus particles has been established [Wakita et al., 2005]. HCVpp or HCVcc are other infectious clones capable of replicating in culture [Lindenbach et al., 2005]. These HCV replication systems can produce virus particles, which are released into the culture medium and re-infect host cells during culture. Analysis of the effects of these systems on PKR expression is also of interest, although it should be noted that the affect by re-infection of HCV may influence outcomes.

It appears that HCV expression and replication are regulated by a number of cellular pathways. In particular, host defense against viruses involves a number of dsRNA-induced proteins, including PKR, TLR-3, and RIG-I. Huh-7 cells harboring HCV replicons respond poorly to dsRNA signaling [Lanford et al.,

2003]. In Huh-7 cells, very limited expression of the Toll like receptor-3 (TLR-3) is observed [Li et al., 2005]. Furthermore, induction of IFN regulatory factor-1 (IRF-1) results in a loss of permissiveness among Huh-7 cells harboring replicons [Kanazawa et al., 2004]. Huh-7.5 cells, known as cured cells, are derived from Huh-7 cells and harbor a single point mutation in RIG-I, the retinoic acid-inducible gene-I, which is thought to participate in sensing the presence of dsRNA molecules [Sumpter et al., 2005]. This Huh-7 derived cell line is highly permissive for HCV replication, however, there may also be disruption of normal antiviral signaling pathways. We therefore examined a BT7-H cell line in addition to a Huh-7 derived cell line. Using our expression system, the response of PKR to HCV replication in HuhT7 cells was less than that of BT7-H cells. Cell lines, such as BT7-H, which supported a higher level of HCV replication appeared to activate PKR more robustly, suggesting that PKR activation by HCV requires adequate HCV expression in the host cell. We therefore speculate that this is due to the formation of HCV dsRNA during replication, along with direct activation of PKR by HCV core protein. Within the Huh-T7 cell line, decreased HCV expression was observed, which appeared to result in decreased PKR activity. As a result, a more dramatic decrease in HCV protein was observed in BT7-H cells, compared to Huh-7 cells. This phenomenon may explain why HCV persists in hepatocytes. Lower levels of HCV replication may result in less PKR activation, thus enabling viral persistence.

In the present study, over-expression of PKR significantly inhibited HCV protein expression in both cell lines. These findings are consistent with an antiviral action of PKR. In Huh-T7 cells, strong PKR expression significantly inhibited HCV expression, while reduced PKR expression permitted HCV expression. To further characterize the relationship between PKR and HCV, we examined the effect of downregulation of PKR. Recently, post-transcriptional gene silencing using siRNA in mammalian cells has been proven an effective means of gene-specific silencing [Elbashir et al., 2001]. We used this method to specifically downregulate PKR. Of note, dsRNA molecules exceeding 30 nucleotides generally induce stimulation of PKR and activate the IFN signaling pathway [Balachandran et al., 2000]. However, siRNA molecules do not because of their short length (19–24 nucleotides). The PKR-specific siRNA used in this study downregulated PKR mRNA, resulting in a 93% reduction of PKR protein expression. Cells in which PKR was downregulated using siRNA demonstrated persistent HCV expression, indicating that PKR is a key molecule in the control of HCV replication and expression.

PKR is thought to be a key antiviral molecule of the IFN signaling pathway [Clemens and Elia, 1997]. However, other reports suggest that translation of HCV is independent of PKR activation by IFN in vitro [Koev et al., 2002]. Recently, several PKR-independent antiviral pathways have been discovered and are believed to play an important role in cellular defense against HCV infection, including 2′5′-oligoadenylate synthetase, MxA, as well as ISG6-16 and ISG15 [Zhu et al., 2003]. Clinically, the level of PKR expression prior to IFN treatment is not predictive of IFN's antiviral effects [MacQuillan et al., 2003; Giannelli et al., 2004]. Thus, it remains a matter of debate whether inhibition of HCV expression by IFN is PKR-dependent. Our results from PKR-silenced cells demonstrating reduced IFN-mediated antiviral effects indicate that IFN's antiviral effects against HCV are at least partially PKR-dependent. These findings suggest that strategies to enhance PKR activity may be a rational therapeutic approach to the management of HCV infection.

Based on these results, we conclude that both HCV expression and IFN effectively induce PKR, and that PKR can influence HCV expression in the presence or absence of IFN. PKR has direct antiviral activity against HCV and is a key mediator of IFN's effects against HCV.

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Anti-*Helicobacter pylori* seropositivity: influence on severity and treatment response in patients with chronic hepatitis C

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SUMMARY. We sought to clarify the incidence and role of *Helicobacter pylori* (*H. pylori*) seropositivity in patients with hepatitis C virus (HCV) infection and the effect of coinfection on interferon-α and ribavirin therapy. The presence of *H. pylori* was tested using a commercially available enzyme immunoassay in serum samples from 93 patients with chronic hepatitis C. Clinical features, HCV markers and response of HCV to interferon-α and ribavirin were compared between *H. pylori*-positive and *H. pylori*-negative patients. Anti-*H. pylori* antibody was detected in 45 (48%) of the 93 patients, whose median HCV-RNA level (495 *vs* 760 kIU/mL; $P = 0.013$) and platelet count (128 *vs* $158 \times 10^3/\mu L$; $P = 0.009$) were significantly lower than in patients with HCV infection alone. Anti-*H. pylori* antibody levels were found to be significantly correlated with fibrosis score ($P = 0.0083$, $r = 0.33$) but inversely related to platelet count ($P = 0.0037$, $r = -0.34$). The sustained response rate for HCV clearance following interferon-α and ribavirin treatment did not differ between patients with and without anti-*H. pylori* seropositivity. The presence of *H. pylori* [odds ratio (OR) 8.61; 95% confidence interval (CI) 1.59–46.70] and fibrosis score (OR 30.13; 95% CI 5.44–166.78) were found by multivariate analysis to be associated with the decrease of platelet count during therapy. Coexistent *H. pylori* infection does not demonstrably influence the clinical course of chronic hepatitis C. A possible connection between *H. pylori* coinfection and thrombocytopenia was found during the treatment course, suggesting that preemptive eradication of *H. pylori* may facilitate completion of treatment and increased sustained virological response.

Keywords: *Helicobacter pylori*, hepatitis C virus, interferon, ribavirin, thrombocytopenia.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of post-transfusion hepatitis and chronic liver disease [1,2]. More than half the patients with acute HCV infections develop chronic hepatitis that leads to liver cirrhosis and/or hepatocellular carcinoma (HCC) in at least 20% of cases [3,4]. Treatment of HCV with interferon (IFN)-α and ribavirin is associated with a sustained response rate of nearly 40% [5,6], which is likely to improve to 55% of treated patients with the use of pegylated IFN-α and ribavirin [7,8].

Helicobacter pylori (*H. pylori*) is a gram-negative bacillus that colonizes the mucous layer of the human stomach. This bacterium has been causally linked with a diverse spectrum of gastrointestinal disorders, including gastritis, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma. Several studies have reported association of *H. pylori* infection to a variety of liver diseases, such as hepatitis A virus, primary biliary cirrhosis and autoimmune hepatitis [9–17]. Patients with chronic liver diseases have also been reported to be significantly more likely to be infected with *H. pylori* than controls. Pellicano *et al.* [11] reported that approximately 90% of 254 patients with HCV-related cirrhosis in Italy were seropositive for anti-*H. pylori* antibodies. However, there is no current evidence as to whether *H. pylori* worsens the course of coexistent hepatitis C. In addition, the effect of *H. pylori* on HCV response to IFN-α and ribavirin is largely unknown.

The objective of this study was to determine the prevalence of anti-*H. pylori* seropositivity in Japanese patients with chronic hepatitis C, the influence of anti-*H. pylori*

*Both authors contributed equally to this study.

Abbreviations: IFN, interferon; HCV, hepatitis C virus; MALT, mucosa-associated lymphoid tissue; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma.

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seropositivity on the clinical, virological and histological characteristics of hepatitis C, and the effect of *H. pylori* co-infection on HCV response to IFN-α and ribavirin therapy.

PATIENTS AND METHODS

Patients

A total of 93 patients with chronic hepatitis C [47 men and 46 women; mean age 58 years (range: 25–74)] who were seen at Shinshu University Hospital and affiliated hospitals of the Nagano Interferon Treatment Research Group between December 2001 and January 2003 were enrolled in the present study. All patients were positive for antibody to HCV (anti-HCV) and positive for HCV-RNA. Diagnosis of chronic hepatitis C was based on the following criteria: (i) persistent elevation of serum alanine aminotransferase (ALT) levels for at least 6 months; (ii) absence of detectable hepatitis B surface antigen; and (iii) exclusion of other causes of chronic liver diseases, such as alcoholic liver injury, autoimmune hepatitis, primary biliary cirrhosis, haemochromatosis, and Wilson disease. No patients had a history of decompensated cirrhosis or HCC, and all were negative for antibody to the human immunodeficiency virus (HIV). Of the 93 patients, 62 had had a liver biopsy prior to IFN and ribavirin combination therapy. Seven of the 93 patients were diagnosed as having cirrhosis. Written informed consent was obtained from each patient.

IFN and ribavirin combination therapy

Interferon-α 2b (Schering-Plough, Tokyo, Japan) was administered at a dosage of 6 million units (MU) daily for 2 weeks, followed by 6 MU three times a week for 22 weeks (total dose, 480 MU). Rivabirin was taken daily for 24 weeks (body weight <60 kg, 600 mg/day; ≥60 kg, 800 mg/day). Patients were followed up for at least 6 months after therapy. For all patients, serum samples were obtained prior to therapy and stored at −70 °C until testing. Additional serum samples were collected just before therapy began, during therapy, immediately after therapy was completed, and 6 months post-therapy.

Serum ALT levels were measured prior to therapy and, in patients undergoing IFN-α and ribavirin therapy, at least once every 4 weeks both during therapy and follow up. Sustained virological responders (SVRs) to IFN and rivabirin therapy were defined as those whose HCV-RNA in serum was undetectable 24 weeks after completing therapy. Non-responders and relapsers who did not meet SVR criteria were defined as non-SVRs.

Necroinflammatory activity was scored according to the histology activity index (HAI) by Knodell *et al.* [18]. The HAI score was determined by combining the scores for portal inflammation (0–4), lobular degeneration and necrosis (0–4), and periportal necrosis (0–10). Fibrosis stage was defined according to the Scheuer fibrosis score: 0, absence; 1, fibrous portal expansion; 2, periportal or portoportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis [19]. Investigators involved in this part of the study were blinded to the results of other portions.

Safety was assessed in this study by recording adverse events reported by patients, clinical laboratory test results (including haematology, blood chemistry and urinalysis), and vital signs.

Serological markers and molecular assays for HCV, HBV, HIV and H. pylori

Anti-HCV antibody, hepatitis B surface antigen and antibody to HIV were measured using commercially available enzyme-linked immunosorbent assays (International Reagents Co., Kobe, Japan). IgG class antibody to *H. pylori* (anti-*H. pylori*) was also detected using an enzyme immunoassay kit (Kyowa Medex Co., Tokyo, Japan). This particular assay has been shown to be an accurate and reliable method for detection of *H. pylori* infection, and is based on high-molecular-weight cell-associated antigens which are highly conserved complexes with important conformational determinants. The enzyme immunoassay kit cutoffs used were those recommended by the manufacturer and were as follows: positive, >2.2 enzyme immunoassay value (EV); indeterminate, 1.8–2.2 EV; and negative, <1.8 EV [20]. In this study, patients positive for anti-*H. pylori* antibodies were defined as >2.2 EV in serum. Patients who did not meet these criteria, including those negative and indeterminate, were regarded as negative for anti-*H. pylori* antibodies. The sensitivity and specificity of this assay were 94.0% and 82.4%, respectively. Serum levels of HCV-RNA were determined using qualitative and quantitative COBAS AMPLICOR assays (Nippon Roche Co. Ltd, Tokyo, Japan), which amplify HCV-RNA by reverse transcription-polymerase chain reaction [21]. HCV genotypes were determined by INNO-LiPA HCV II (Innogenetics, Gent, Belgium). ALT, and other relevant biochemical tests, were performed using standard methods.

Statistical analysis

Mann-Whitney *U*-test was used to analyse continuous variables. Chi-squared test with Yates' correction was used for the analysis of categorical data. Pearson's correlation coefficient was used to evaluate the relationships between the titre of anti-*H. pylori* antibodies and platelet count or fibrosis score. Multivariate analysis was performed using a logistic regression model with a stepwise method. A *P*-value of ≤0.05 was considered significant. Statistical analyses were performed using SigmaStat (version 2.03, SPSS Inc., Chicago, IL, USA) and SPSS 6.1J (SPSS Inc.).

RESULTS

Detection of H. pylori

Anti-*H. pylori* antibody was detected in 45 of 93 (48%) patients. The median serum anti-*H. pylori* antibody and HCV-RNA levels were 1.7 EV (range, 0.4–7.0) and 686 KIU/mL (range, 6.1–850), respectively. Clinical and virologic features were compared between patients with and without anti-*H. pylori* antibodies in Table 1. A history of blood transfusion was more common in patients with anti-*H. pylori* antibodies than in those without anti-*H. pylori* antibodies (53% *vs* 29%; $P = 0.022$). The median platelet count in patients with *H. pylori* was significantly lower than that in those without *H. pylori* (128 *vs* 158 $\times 10^3/\mu L$; $P = 0.009$) at the onset of treatment. Additionally, median levels of HCV-RNA in serum were significantly lower in patients with anti-*H. pylori* antibodies (495 *vs* 760 KIU/ml; $P = 0.013$) (Table 1). Platelet count and fibrosis score were examined for their correlation with levels of anti-*H. pylori* IgG. The titre of anti-*H. pylori* antibodies was significantly correlated with fibrosis score ($P = 0.0083$, $r = 0.33$) though inversely related with platelet count ($P = 0.0037$, $r = -0.34$) (Fig. 1). The rate of HCV response to IFN-α and ribavirin therapy did not differ between patients with and without anti-*H. pylori* antibodies (Table 1).

Effect of H. pylori infection on HCV response to IFN-α and ribavirin therapy

Of the 93 patients receiving IFN-α and ribavirin therapy, 42 (45%) were SVRs. There was no significant difference between SVRs and non-SVRs according to sex, history of blood transfusion, or median ALT level at the start of therapy. SVRs were found more frequently in younger patients ($P = 0.032$) (Table 2). Prior to treatment, median HCV-RNA level in the SVR group [460 KIU/mL (range 6.1–850)] was significantly lower than that in the non-SVR group [718 KIU/mL (range 140–850), $P = 0.022$]. Sustained viral clearance in patients with genotypes 2a or 2b was higher than in those with genotype 1b ($P < 0.001$). Sustained HCV response to IFN-α and ribavirin therapy did not differ between patients with and without *H. pylori* infection. In addition, the titre of anti-*H. pylori* antibodies in serum did not influence response to antiviral therapy (Table 2).

To assess whether *H. pylori* decreased platelet count during IFN-α and ribavirin therapy, we compared the clinical features of patients who did or did not have a decreased platelet count ($<70 \times 10^3/\mu L$). Complete data at the start of therapy were available in 69 patients. Of these, six of seven patients with cirrhosis had decreased platelet count. Using univariate analysis, anti-*H. pylori* positivity, HCV viral load and fibrosis score were associated with a decreased platelet count during combination therapy (data not shown).

Table 1 Clinical features of *Helicobacter pylori*-positive and -negative patients with chronic hepatitis C

	Anti-*H. pylori*		
	Positive ($n = 45$)	Negative ($n = 48$)	*P*-value
Age (years)	58 (44–74)	60 (25–73)	0.16*
Male, *n* (%)	26 (58)	22 (46)	0.36†
History of BTF, *n* (%)	24 (53)	14 (29)	0.022†
ALT (IU/L)	105 (14–693)	100 (19–286)	0.63*
Haemoglobin (g/dL)	14.3 (11.3–17.9)	14.4 (12.6–17.8)	0.30*
Platelet ($\times 10^3/\mu L$)	128 (81–317)	158 (70–200)	0.009*
Fe (μg/mL)	149 (23–278)	143 (43–291)	0.13*
Ferritin (ng/mL)	110 (8.4–700)	130 (2.5–1300)	0.40*
HAI score‡	12 (4–18)	13 (6–18)	0.25*
Fibrosis score‡	2 (0–4)	2 (0–4)	0.11*
HCV genotype, *n* (%)			
1b	32 (71)	27 (56)	0.19†
2a or 2b	13 (29)	21 (44)	
HCV RNA level (KIU/mL)	495 (38–850)	760 (6.1–850)	0.013*
Sustained HCV response to IFN-α and ribavirin (%)	20 (44)	22 (46)	0.89†

Data are median (range) unless otherwise specified. ALT, alanine aminotransferase; BTF, blood transfusion; HCV, hepatitis C virus; HAI, histology activity index; IFN-α, interferon-α.
*Mann–Whitney *U*-test; †chi-squared test.
‡Liver biopsy data were available in 62 patients.

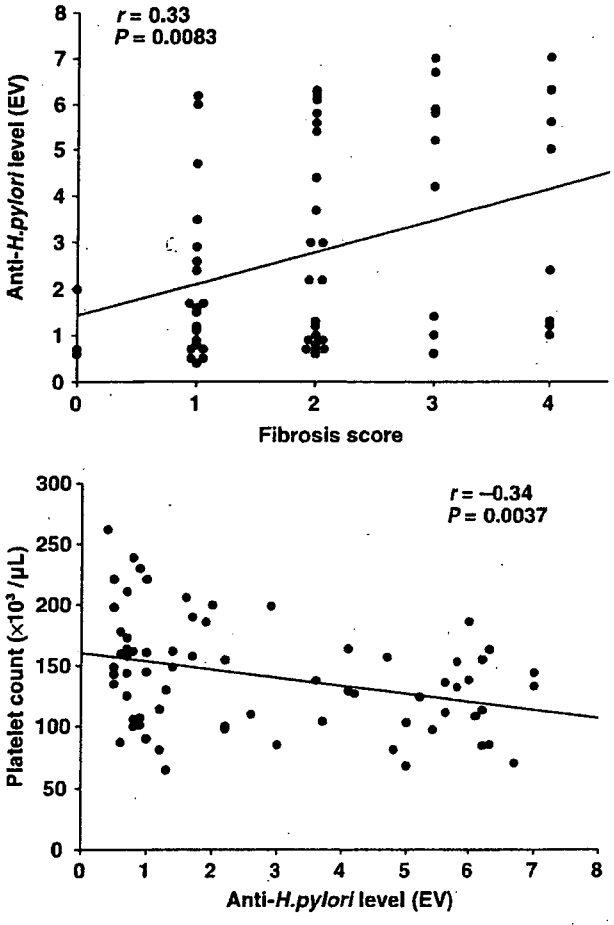

Fig. 1 Influence of anti-*Helicobacter pylori* antibody titre on clinical characteristics in patients with chronic hepatitis C. The titre of anti-*H. pylori* antibody was significantly correlated with fibrosis score (a; $r = 0.33$, $P = 0.0083$) and inversely correlated with platelet count (b; $r = -0.34$, $P = 0.003$.

Table 3 Multivariate analysis of factors associated with thrombocytopenia during interferon-α and ribavirin therapy in 69 patients with chronic hepatitis C

Factor	*n*	Odds ratio (95% CI)	*P*-value
Age (years)			
≥65	28	1.00	
55–64	26	0.11 (0.01–0.83)	0.033
≤54	15	0.66 (0.08–5.59)	0.70
ALT (IU/L)			
<110	42	1.00	
≥110	27	4.04 (0.86–18.91)	0.077
Helicobacter pylori			
Negative	37	1.00	
Positive	32	8.61 (1.59–46.70)	0.013
Fibrosis score			
0–2	19	1.00	
3	25	4.84 (0.65–36.13)	0.12
4	7	30.13 (5.44–166.78)	0.0053
Unknown	18	0.92 (0.10–8.46)	0.94

CI, confidence interval; ALT, alanine aminotransferase.

Table 3 summarizes the multivariate analysis of factors possibly influencing the decrease of platelet count during therapy. Of these, presence of *H. pylori* [odds ratio (OR) 8.61; 95% confidence interval (CI) 1.59–46.70], and fibrosis score (OR 30.13; 95% CI 5.44–166.78) were found to be associated with a decreased platelet count.

DISCUSSION

In this study, we found that approximately 50% of Japanese patients with chronic hepatitis C were coinfected with *H. pylori*. This anti-*H. pylori* seroprevalence is similar to our

Table 2 Hepatitis C virus (HCV) response to interferon-α and ribavirin therapy in relation to HCV viral load, genotype and *Helicobacter pylori* status

	Sustained response	No sustained response	*P*-value
n	42	51	
Age (years)	56 (25–74)	61 (42–73)	0.032*
Male, *n* (%)	22 (52)	25 (49)	0.75†
Platelet ($\times 10^3/\mu L$)	145 (81–317)	134 (65–239)	0.15*
ALT (IU/L)	106 (33–693)	99 (14–346)	0.38*
HCV-RNA level (KIU/mL)	460 (6.1–850)	718 (140–850)	0.022*
HCV genotype 1b, *n* (%)	18 (43)	41 (80)	<0.001†
HCV genotype 2a or 2b, *n* (%)	24 (57)	10 (20)	
H. pylori positive, *n* (%)	20 (48)	25 (49)	0.89†
Titre of anti-*H. pylori* (EV)	1.9 (0.4–7.0)	1.6 (0.5–7.0)	0.95*

Data are median (range) unless otherwise specified. ALT, alanine aminotransferase.
*Mann–Whitney *U*-test; †chi-squared test.

recent report on healthy individuals in Japan [10]. Although the urea breath test is more specific for an active *H. pylori* detection, the treatment arm of this study was designed to measure HCV response to IFN-α and ribavirin therapy. Further study using the urea breath test is needed to clarify the exact frequency of *H. pylori* in chronic patients.

Concerning disease severity, it has been reported that dual infection with HCV and either hepatitis A virus or hepatitis B virus was associated with more severe and rapidly progressive liver disease [22–24]. However, no evidence was found to suggest that *H. pylori* increases the severity of chronic hepatitis C, because clinical and biochemical evaluations, notably ALT levels, did not differ greatly between patients with HCV infection alone and those coinfected with HCV and *H. pylori*. This phenomenon comparable with our prior reports on single and coinfection of HCV and hepatitis G and SEN viruses [25–29].

Several reports have demonstrated viral interference between hepatitis B virus and HCV [23,30,31], and that increasing the replication of one agent can diminish replication of the other. Although there is no evidence of interference between HCV and this bacterium in literature, the HCV-RNA titre in patients with HCV and *H. pylori* coinfection was significantly lower than in patients with HCV infection alone ($P = 0.013$), suggesting that *H. pylori* infection might interfere with HCV replication. At present, we can only describe the observation of a possibility of viral–bacterium interference and cannot provide a sound scientific basis for its occurrence. Additional studies, for instance HCV replicon system analysis *in vitro*, would be required to validate this result. While HCV genotype and pre-treatment HCV-RNA level were seen to be significantly associated with HCV treatment response in this and prior studies [32,33], there was no significant difference in the sustained HCV treatment response between those who had HCV infection alone and those with HCV and *H. pylori* coinfection.

Over the past few years, *Helicobacter* species have been found to be present in the liver of HCV-negative patients, and have been associated with HCC development in the non-cirrhotic liver [14,34,35]. Rocha *et al.* [16] have recently reported that virtually all patients with HCC are *Helicobacter* species positive in their HCC, and 61–68% of those with cirrhosis are *Helicobacter* positive in liver tissue, compared with 4.5% and 3.2% of hepatitis patients and controls, respectively. This suggests that the presence of *Helicobacter* species DNA sequences in the liver may be a co-risk factor in the progression of chronic HCV liver disease. In this study, we demonstrated a strong correlation between the titre of anti-*H. pylori* antibodies and the degree of fibrosis ($P = 0.0083$; $r = 0.33$). However, since no patients with HCC were enrolled, we were unable to assess whether the level or positivity of anti-*H. pylori* antibodies was associated with the development of HCC. As there are no reports to clarify the clinical significance of anti-*H. pylori* antibody level in patients with liver disease, this significant finding should be expanded in larger populations that contain patients with chronic hepatitis and cirrhosis.

Recently, *H. pylori* has been suspected to be involved in various autoimmune disorders, including idiopathic thrombocytopenia [36,37]. Several studies have also reported that the eradication of *H. pylori* is often accompanied by a significant increase in platelet count in patients with idiopathic thrombocytopenia [37,38]. Although this clinical observation suggests the involvement of *H. pylori*, little is known about the pathogenesis of *H. pylori*-associated idiopathic thrombocytopenia. We found that serum platelet count was significantly lower in patients with HCV/*H. pylori* coinfection in this study as well, and observed an inverse relation between anti-*H. pylori* antibody titre and platelet count ($P = 0.0037$). Thrombocytopenia is a major haematologic disorder commonly observed in patients with liver cirrhosis. Splenic sequestration of platelets, impaired platelet production from insufficient thrombopoietin secretion, and anti-GPIIb-IIIa autoantibody-mediated platelet destruction have been proposed to be associated with thrombocytopenia in patients with cirrhosis [39–41]. Our results raise the possibility that *H. pylori* seropositivity might contribute to thrombocytopenia in patients with HCV infection. It is possible, however, that the low platelet counts might have been an indirect consequence of an autoimmune reaction in patients with HCV infection, and may not necessarily indicate the presence of the *Helicobacter* species in the liver. Using multivariate analysis (Table 3), fibrosis score and the presence of *H. pylori* were significantly associated with a decrease in platelet count during IFN and ribavirin therapy. Hence, it might be useful to eradicate H. pylori prior to therapy, which would presumably increase platelet count and decrease the rate of reduction or cessation of IFN. As the treatment arm of the study was initially designed to measure HCV response only, eradication of *H. pylori* had not been performed. Eradication of *H. pylori* in patients with chronic hepatitis C prior to IFN and ribavirin therapy is currently being planned in a forthcoming study.

In conclusion, coexistent *H. pylori* infection does not influence the clinical course of hepatitis C, but might interfere with HCV replication. The presence of *H. pylori* is associated with decreased of platelet count during IFN-α and ribavirin therapy, indicating that preemptive eradication of *H. pylori* may facilitate completion of treatment and an increase of sustained viral response.

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A Genomewide DNA Microsatellite Association Study of Japanese Patients with Autoimmune Hepatitis Type 1

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Genetic predisposition to type 1 autoimmune hepatitis (AIH) is linked mainly to HLA class II genes. We previously searched the whole HLA region for AIH susceptibility genes using microsatellite markers and found only HLA-DR/DQ to be a candidate region for this suspected multifactorial disease. As such, the aim of this study was to broaden our search and screen the whole genome for additional genes that might contribute to type 1 AIH susceptibility. Eighty-one patients with type 1 AIH (15 men, 66 women, average age 55.9) and 80 healthy sex- and age-matched Japanese controls were enrolled in this study. We performed a case-control association study using 400 polymorphic microsatellite markers with an average spacing of 10.8 cM distributed throughout the whole genome. Two markers, one on chromosome 11 (D11S902, Pc = 0.013) and one on chromosome 18 (D18S464, Pc = 0.008), were revealed to have statistically significant associations with AIH. An additional 7 markers (D2S367, D6S309, D9S273, D11S1320, D16S423, D17S938, and D18S68) were also found to be candidate susceptibility regions. In addition, our results showed there were 17 regions that may contain genes of resistance to AIH. No specific markers were detected in HLA-DR4-negative patients, and no differences were seen in the clinical courses of patients (severe versus mild to moderate). *Conclusion:* This first genomewide scan of Japanese AIH patients revealed at least 26 candidate AIH susceptibility or resistance regions other than HLA class II loci. These results also suggested that the products of several genes interact to determine heritable susceptibility to AIH. (HEPATOLOGY 2007;45:384-390.)

Autoimmune hepatitis (AIH) is a chronic active hepatitis of unknown etiology characterized by hypergammaglobulinemia and autoantibodies; genetic and environmental factors are suspected to be important in its pathogenesis.[1-3] Several studies from ethnically different countries have clarified strong genetic bases for both the susceptibility to and behavior of AIH.[4-14] Among Caucasians susceptibility to developing type 1 AIH specifically is associated with the DRB1*0301 and DRB1*0401 alleles[6-10] and among Japanese with the DRB1*0405 allele[11] at the HLA class II DRB1 locus, which encodes a polymorphic β chain in the HLA-DR antigen. However, the association of these DRB1 antigens with susceptibility to developing type 1 AIH is not complete because not all AIH patients possess these antigens. This suggests that additional susceptibility genes may contribute to the development of type 1 AIH. Moreover, there may be resistance genes, which may protect against development of the disease. Whereas previously candidate susceptibility and resistance genes were searched for on an individual level in Caucasian patients with AIH,[15,16] the current study searched for them comprehensively throughout the whole genome.

Microsatellites (also called short tandem repeat polymorphisms) are tandem arrays of short stretches of noncoding nucleotide sequences that are usually repeated between 15 and 30 times.[17] The obvious advantages of microsatellites are high heterozygosity, ubiquity throughout the genome, and PCR typability. Association analysis using microsatellite markers is a powerful yet cost-efficient method for mapping candidate susceptibility genes

Abbreviations: AIH, autoimmune hepatitis; PT, prothrombin time; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; PBC, primary biliary cirrhosis.

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Table 1. Statistically Significant Alleles Associated with AIH

Chromosome	Marker	Significant Allele	AIH (n = 81), %	Control (n = 80), %	OR	χ^2	P	Pc
2p22.3	D2S367	312	11.1	0	17.69	7.93	0.0049	0.068
6p24.3	D6S309	312	28.4	10.4	3.40	7.31	0.0069	0.082
9q21.11	D9S273	215	19.8	4.5	5.25	7.65	0.0057	0.085
11p15.1	D11S902	156	28.4	7.5	4.92	10.47	0.0012	0.013
11q25	D11S1320	267	96.3	85.1	4.56	5.76	0.0164	0.082
16p13.3	D16S423	135	53.1	31.3	2.48	7.06	0.0079	0.094
17p13.2	D17S938	252	28.4	10.4	3.40	7.31	0.0069	0.062
18p11.22	D18S464	304	25.9	6.0	5.51	10.40	0.0013	0.008
18q21.33	D18S68	287	9.9	0	15.61	7.00	0.0082	0.082

Abbreviations: OR, odds ratio; Pc, corrected P.

in multifactorial genetic diseases.[18] If the frequencies of microsatellite markers differ significantly between patients and controls, there may be susceptibility or resistance genes may be near them, which can be further analyzed by sequencing. We previously screened the HLA region for AIH susceptibility genes using this method and found only HLA-DR/DQ to be a candidate region.[19] To search for additional genes influencing the development of type 1 AIH in the whole genome, we performed an association analysis using 400 microsatellite markers spaced an average of 10.8 cM apart.

Patients and Methods

Subjects. Eighty-one patients with type 1 AIH (15 men, 66 women, average age 55.9 years) and 80 healthy sex- and age-matched Japanese controls were enrolled in this study. Seventy-seven of the 81 patients had been included in our previous study that scanned the HLA region.[19] All subjects were residents of Nagano Prefecture, Japan, and their racial background was Japanese. Of the 81 patients, 10 were probable cases of AIH (score of 14-17 after treatment) and 71 were definite cases, according to the scoring system of the International Autoimmune Hepatitis Group.[20] Ten patients had slightly elevated titers of ANA (40×), and 69 patients had high titers of ANA (more than 80×). ANA was not found in 2 of the patients, though they were both positive for anti–smooth muscle antibodies (80×). The patients were classified as having type 1 AIH based on antibody profiles. No viral markers, such as hepatitis B surface antigen, anti-hepatitis C virus antibody (second and third generations), or hepatitis C virus RNA, were detected in the serum. This study was approved by the Ethics Committee of the Shinshu University School of Medicine. Written informed consent was obtained from each subject.

DNA Preparation. Genomic DNA from patients and controls was isolated by phenolic extraction of sodium dodecyl sulfate–lysed and proteinase K–treated cells as described previously.[19,21]

HLA Typing. HLA classes I and II alleles were determined using a Micro SSP™ DNA Typing Kit (One Lambda, Canoga Park, CA). DNA typing of the DRB1 and DQB1 alleles was performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis as previously described.[19,21]

Microsatellite. Fluorescence-tagged primers for 400 microsatellite markers that defined a 10.8-cM resolution human index map (ABI PRISM Linkage Mapping Set Version 2.5 MD10) were purchased from Applied Biosystems (Foster City, CA). PCR was performed following the manufacturer's instructions. PCR-amplified products were denatured for 5 minutes at 100°C, mixed with formamide-containing stop buffer, and then electrophoresed on a 4% polyacrylamide denaturing gel containing 8 M urea in a Model 377 automated DNA sequencer (Applied Biosystems). Fragment sizes were determined automatically by GeneScan software (Applied Biosystems) as described previously.[19]

Statistical Analysis. Phenotypic frequency at polymorphic sites in the 400 microsatellites was estimated by direct counting. The significance of differences between patients and controls in allele distribution was tested by the χ^2 method with continuity correction. The P value was corrected by multiplication by the number of alleles observed in each locus tested (corrected P value: Pc value). A Pc value of less than 0.1 was considered statistically significant.

Results

Four hundred microsatellite markers were used in a genomewide linkage search in AIH patients with age- and sex-matched controls to localize genetic intervals that might contain AIH susceptibility or resistance loci. This search revealed several candidate susceptibility and resis-

Table 2. Statistically Significant Alleles Associated with Resistance to AIH

Chromosome	Marker	Significant Allele	AIH (n = 81), %	Control (n = 80), %	OR	χ^2	P	Pc
1p13.1	D1S252	104	2.4	14.9	0.14	7.64	0.0057	0.051
1q42.2	D1S2800	217	2.4	14.9	0.14	7.64	0.0057	0.057
1q41	D1S2785	173	12.3	29.8	0.33	6.95	0.0084	0.084
3q28	D3S1580	225	8.6	25.4	0.28	7.56	0.0060	0.090
4p14	D4S405	295	27.1	49.2	0.38	7.66	0.0056	0.062
4q21	D4S2964	127	9.8	25.4	0.32	6.27	0.0123	0.086
5p14	D5S641	313	24.7	46.2	0.38	7.56	0.0060	0.072
5q35.1	D5S400	229	17.3	40.3	0.31	9.70	0.0018	0.018
7q22.1	D7S515	170	39.5	67.2	0.32	11.24	0.0008	0.011
7q32.1	D7S530	119	3.7	17.9	0.18	8.13	0.0044	0.052
8p21.2	D8S1771	359	2.5	14.9	0.14	7.64	0.0057	0.046
10p15.3	D10S249	119	19.8	41.8	0.34	8.52	0.0035	0.039
10p13	D10S1653	126	6.2	20.9	0.25	7.10	0.0077	0.092
14q22.3	D14S276	235	0	10.4	0.00	8.88	0.0029	0.032
15q12	D15S1002	120	7.4	25.4	0.24	9.02	0.0027	0.032
15q13.3	D15S165	186	90.1	100	0.00	7.00	0.0082	0.065
17p13.3	D17S849	261	25.9	46.3	0.41	6.66	0.0099	0.049

Abbreviations: OR, odds ratio; Pc, corrected *P*.

tance regions throughout the genome (Tables 1 and 2). Strong evidence for linkage was detected by marker D11S902 (28.4% vs. 7.5%, Pc = 0.013) on chromosome 11p15.1 (Fig. 1A) and D18S464 (25.9% vs. 6.0%, Pc = 0.008) on chromosome 18p11.22 (Fig. 1B). An additional 7 markers (D2S367, D6S309, D9S273, D11S1320, D16S423, D17S938, and D18S68) were also found to be candidate susceptibility regions (Table 1). We found 17 additional regions in which there might be genes that confer resistance to AIH (Table 2). We used the National Center for Biotechnology Information Map Viewer, National Library of Medicine, National Institute of Health (http://www.ncbi.nlm.nih.gov/mapview/; Table 3) to identify several candidate

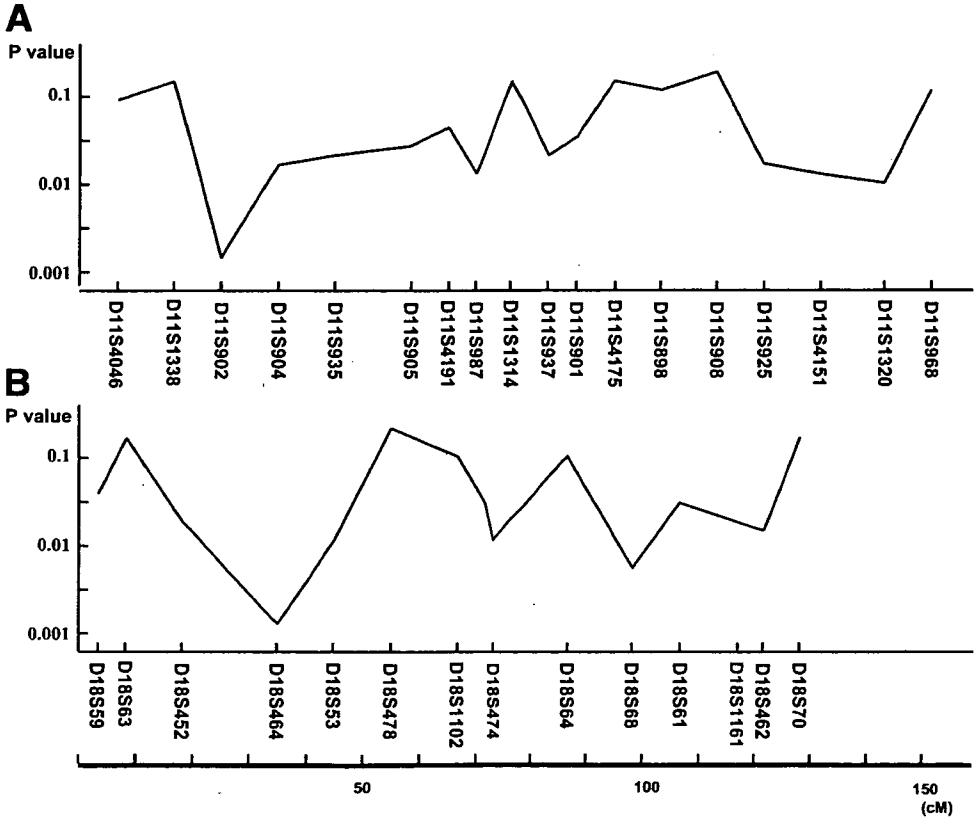

Fig. 1. AIH susceptibility gene mapping by association analysis on (A) chromosome 11 and (B) chromosome 18. *P* values of association between control and patient groups are displayed with the location of microsatellite markers used for mapping.