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Possible Molecular Mechanism of the Relationship Between NS5B Polymorphisms and Early Clearance of Hepatitis C Virus During Interferon Plus Ribavirin Treatment

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We previously reported the relationship between viral polymerase polymorphisms and the initial decline in viral load induced by interferon-α and ribavirin therapy in genotype 1b-related chronic hepatitis C patients. The presence of E124K and I85V of NS5B was closely associated with viral clearance at 8 weeks of treatment. The aim of this study was to investigate the mechanisms by which this polymorphism of NS5B protein affects early viral clearance. We used a replicon system derived from strain O, genotype 1b virus. Three mutants (V85I), (K124E), and (V85I/K124E) were introduced to the replicon. OR6c, a derivative of HuH7 cells, was transfected with the replicon including a luciferase reporter gene. Luciferase activities were measured 72 hr post-transfection. All three mutants showed higher luciferase activity than that of the wild type, and the V85I mutant showed the highest activity. This result was also confirmed by neomycin gene-containing replicons with same mutations. All replicons were down-regulated by ribavirin, but the level of reduction in the V85I mutant was the lowest. Our results suggested that this mutation at least partly contributes to resistance to early viral clearance during interferon and ribavirin combination therapy. ***J. Med. Virol. 80:632–639, 2008.***

KEY WORDS: hepatitis C virus; NS5B polymorphism; replicon; interferon and ribavirin combination therapy; viral proliferation

INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health. An estimated 65–80% of the individuals infected with HCV develop persistent infection while 20–50% develop cirrhosis and 5% develop hepatocellular carcinoma (HCC) [Liang et al., 2000; Gao et al., 2004]. Until recently, interferon (IFN)-α and IFN-β were the only available treatments for HCV infection, although only 10–15% of treated subjects achieved sustained viral eradication with IFN monotherapy, and early viral clearance after initiation of IFN monotherapy was correlated with sustained viral clearance [Saito et al., 2000].

The current approved treatment for HCV infection is pegylated IFN-α (peg-IFN) in combination with ribavirin (RBV). This combination therapy leads to viral clearance in 50–80% of cases, depending on the infecting HCV genotype, and 50% of patients with HCV genotype 1b and high baseline levels of viral RNA do not achieve a sustained virological response with the combination therapy after 48 weeks [Manns et al., 2001; Fried et al., 2002; Feld and Hoofnagle, 2005]. Several prior studies have attempted to predict the efficacy of IFN plus RBV combination therapy. A quantitative measurement of HCV viremia or the initial decline in viral load is a reliable marker for early prediction of the therapeutic response to IFN and RBV combination therapy [Zeuzem et al., 1998; Bouvier-Alias et al., 2002; Takahashi et al., 2005; Lukasiewicz et al., 2007].

RBV is a broad-spectrum nucleoside analogue antiviral drug which is especially noted for its actions

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against RNA viruses and exhibits in vitro activity against some DNA and RNA viruses, including certain members of *Flaviviridae* [Sidwell et al., 1972]. It has recently been demonstrated that the antiviral activity of RBV can result from the ability of a viral RNA-dependent RNA polymerase (RdRP) to utilize RBV triphosphate and to incorporate this nucleotide into the viral genome with reduced specificity, thereby mutagenizing the genome and decreasing the yield of infectious virus [Crotty et al., 2000; Lanford et al., 2003]. Moreover, RBV exhibits an antiviral effect through a mechanism of error-prone replication in the HCV subgenomic replication system [Contreras et al., 2002]. Although RBV by itself cannot decrease serum HCV RNA levels in patients, it has been demonstrated that combination therapy with RBV and either IFN-α or peg-IFN yields a higher sustained response rate than is achieved with IFN-α monotherapy [Pol et al., 2000; Poynard et al., 2000; Saracco et al., 2001].

We previously reported the relationship between viral RdRP polymorphisms and the initial decline in viral load induced by IFN-α and RBV therapy in genotype 1b-related chronic hepatitis C patients [Kumagai et al., 2004]. Substitution of glutamic acid to lysine at the 124th position (E124K) and of isoleucine to valine at the 85th position (I85V) of NS5B was closely associated with viral clearance at 8 weeks of treatment.

In this study, we used the genotype 1b HCV replicon system [Ikeda et al., 2005] to generate NS5B mutants (E124K, I85V, and both) and we compared the replication activity with that of the wild-type replicon and to analyze how this polymorphism of NS5B protein affects early viral clearance during combination therapy with IFN and RBV. We also examined the significance of NS5B polymorphisms in the RBV-induced decrease in viral replication. We concluded that the identified polymorphism of NS5B partly affects viral replication.

MATERIALS AND METHODS

Cell Culture System

OR6 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal calf serum, penicillin, and streptomycin (complete DMEM), in addition to G418 (300 μg/ml; Geneticin, Invitrogen), and were then passaged twice a week at a 5:1 split ratio. OR6c cells are cured OR6 cells from which genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described [Ikeda et al., 2005].

Plasmids

The plasmids pON/C-5B/KE (Fig. 1a) and pHCV-O were described previously [Ikeda et al., 2005]. This plasmid includes the adaptive mutation of K1609E of NS3 to enhance the efficiency of replication, this adaptive mutation was reported by Lohman et al. (22) The plasmid pON/C-5B/KE contains neomycin phosphotransferase (Neo) downstream of HCV IRES and the full length HCV-O polyprotein coding sequence downstream of encephalomyocarditis virus (EMCV) IRES. To introduce a pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/KE/(V85I&K124E), we first made PCR fragments including the partial NS5B region with the primers 5′-ggatcccgatctcagcgacgg-3′ and 5′-tctagaggtcccattcgccattac-3′. This 2.4-kb fragment was subcloned into pSTBlue1 Blunt vector (Novagen, Madison, WI) to generate pSTBlue-1MN002. Each vector expressing the V85I mutant, K124E mutant, and V85I&K124E double mutant of HCV-O was generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) to generate pSTBlueMN002(V85I), pSTBlueMN002(K124E) pSTBlueMN002(V85I&K124E). Next, pON/C-5B was

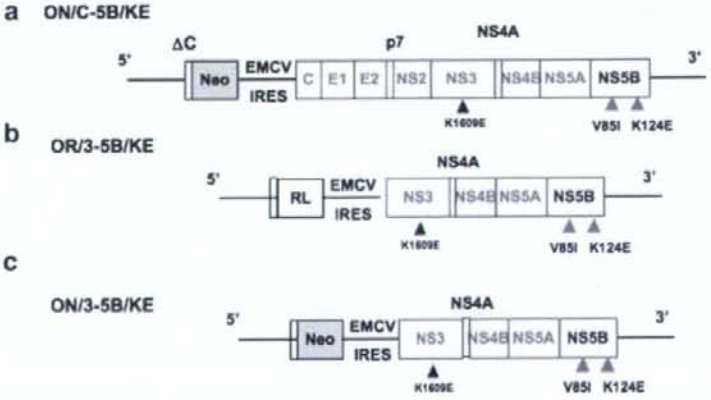

Fig. 1. **a**: Organization of genome-length HCV RNA derived from HCV-O. Open reading frames, untranslated regions, EMCV IRES, and Neo genes are depicted as shaded boxes, thin lines, thick lines, and open boxes, respectively. ΔC indicates the 12 N-terminal amino acid residues of the core as a part of IRES. This construct also contains adaptive mutation K1609E which is indicated by a black triangle. We use this construct as a wild type. Grey triangle is the position of 85 and 124 in NS5B which we generated mutation to the replicon for this experiments. **b**: The construct of the reporter subgenomic HCV replicon carries the renilla luciferase gene (RL). **c**: The construct of the reporter subgenomic HCV replicon carries the Neo gene.

digested with *Sna*b1 and *Xba*1 and subcloned into pSTBlue-1 to create pSTBlueMN001. All of the pSTBlue-MN002mutants were digested with *Bam*H1 and *Xba*1, which were subcloned into pSTBlueMN001 to create pSTBlueMN001mutant. The pSTBlueMN001 mutants were digested with *Sna*b1 and *Xba*1 and re-ligated in pON/C-5B/KE to introduce pON/C-5B/KE/(V85I), pON/C-5B/KE/(K124E), pON/C-5B/(V85I&K124E). The plasmids pOR/3-5B/KE/(V85I), pOR/3-5B/KE/(K124E) and pOR/3-5B/KE/(V85I&K124E), were constructed from pOR/3-5B/KE (Fig. 1b) by swapping for fragments of pSTBlueMN001 mutants digested with *Sna*b1 and *Xba*1. DNA sequencing of the manipulated regions of the plasmids verified all mutations.

RNA Transfection and Selection of G418-Resistant Cells

For electroporation, OR6c cells were washed twice with ice-cold phosphate buffered saline (PBS) and resuspended at 10^7 cells/ml in PBS. Twenty microgram of ON/C-5B/KE or its mutant derived RNA was mixed with 500 μl of the cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad, Hercules, CA). The mixture was immediately subjected to two pulses of current at 1.2 kV, 25 μF, and maximum resistance. Following 10 min of incubation at room temperature, cells were seeded into 10-cm dishes. Cells were selected in complete DMEM with 300 μg/ml G418. About 3 weeks after transfection and G418 selection, cells were fixed and stained with Coomassie brilliant blue (0.6 g/l in 50% methanol–10% acetic acid) and the number of colonies was counted.

Transient-Replication Assays With Luciferase Replicons

OR6c cells were transfected by electroporation as the same protocol described above using 20 μg of OR/3-5B/KE or its mutants derived RNAs carrying the renilla luciferase (RL) gene. After addition of 2 ml of complete DMEM, 2×10^4 of aliquot OR6c cells were plated in 24-well plates at least in triplicate for each assay and harvested at various time points with renilla lysis reagent (Promega KK, Tokyo, Japan) and subjected to the RL assay according to the manufacturer's protocol (Promega). Values obtained with cells harvested 6 hr after electroporation were used to correct for the transfection efficiency.

IFN and Ribavirin Treatment

To monitor the anti-HCV effect of IFN and RBV on replication, OR6c cells were transfected by electroporation using 10 μg of OR/3-5B/KE derived RNAs as described elsewhere [Crotty et al., 2000]. OR6c cells (2×10^4/well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 hr. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, and 20 units/ml or RBV at a final concentration of 50,100, and 200 μM for 72 hr, harvested with renilla lysis reagent (Promega), and assayed for luciferase activity according to the manufacturer's protocol. We also studied about the additional effect of RBV (100 μM) on IFN (1 u/ml).

Cell Viability

We checked toxic effect of IFN and RBV. Effect of IFN (1 and 4 units/ml), and RBV (50 and 100 μM) on cell viability was investigated. To examine the cytotoxic effect of IFN and RBV on OR6c cells with OR/3-5B/KE replicon RNA, the cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24-hr culture, the cells were treated with IFN or RBV at final concentrations of 2 and 4 units/ml or 50 and 100 μM, respectively, in the absence of G418. After incubation for 72 hr, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen) treatment.

Indirect Immunofluorescence

Cells were grown on four-well chamber slides until 70–80% confluent, washed three times with PBS, and fixed in methanol–acetone (1:1, v/v) for 10 min at room temperature. Dilutions of primary murine monoclonal antibody to residues 21–40 of the core protein (2Zcp11; Tokushu Men-eki Institute, Tokyo) (1:1,000), were prepared in PBS containing 3% bovine serum albumin and incubated with fixed cells for 2 hr at room temperature. After additional washes with PBS, specific antibody binding was detected with a goat anti-mouse immunoglobulin G-fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Cells were washed with PBS, and mounted in DAKO Fluorescent mounting medium (DAKO Japan, Tokyo, Japan) prior to examination using a Zeiss AxioPlan2 fluorescence microscope.

Statistical Analysis

Difference in relative luciferase activity among mutant replicons and differences in anti-HCV activity of RBV among mutant replicons were tested using Student's *t*-test and Mann–Whitney *U*-test as appropriate. *P*-values <0.05 were considered statistically significant.

RESULTS

Mutation in NS5B Enhances Levels of Replication on Transient Assay

To investigate whether the mutations in NS5B of the HCV genome affect replication, we used subgenomic HCV replicons with the renilla luciferase gene for transient assay [Ikeda et al., 2005]. In a previous study [Kumagai et al., 2004], substitution of glutamic acid at the 124th position with lysine and substitution of isoleucine at the 85th position with valine in NS5B yielded a complete match with the population of good

responders (5 out of 5 patients). We introduced mutations to two different types of replicon to obtain ON/C-5B/KE(V85I), ON/C-5B/KE/(K124E), ON/C-5B/KE (V85I&K124E), OR/3-5B/KE/(V85I), OR/3-5B/KE(K124E), and OR/3-5B/KE(V85I&K124E) as described in Materials and Methods Section. The subgenomic replicons with V85I showed higher replication activity than the wild-type replicon in OR6c cells (Fig. 2). Also the replicon with K124E and the replicon with V85I&K124E showed slightly higher replication activity than the wild type, but the replicon with K124E single amino acid mutation did not show statistically higher replication activity than the wild type (Fig. 2). We initially expected that double mutations (V85I&K124E) would lead to better replication than either of the single mutations (V85I or K124E), but interestingly the V85I mutation on NS5B replicated best. This result indicated that the level of replication was affected by amino acid substitution at the 85th position.

Mutation in NS5B Enhances the Efficiency of Colony Formation in Cured Cells

In colony formation assay, we used cured subgenomic replicon cells (OR6c), since cured cells enhanced colony formation of the replicon more efficiently than did parental HuH-7 cells. We examined the effect of these mutations in full-length replicon, ON/C-5B/KE by a colony formation assay. In the initial experiment, we introduced each 20 μg of RNA derived from the ON/C-5B/KE, ON/C-5B/KE/(V85I), ON/C-5B/KE/(K124E), and ON/C-5B/KE/(V85I&K124E) into OR6c cells. After 3 weeks of G418 selection at a concentration of 300 μg/ml, only one colony was obtained and the same result was obtained with ON/C-5B/KE(K124E) and ON/C-5B/KE/(V85I&K124E) transcripts. In repeated experiments, the number of G418-resistant colonies was reproducibly one or zero, but when ON/C-5B/KE/(V85I) transcripts was electroporated, G418 resistant 4–6 colonies was obtained in repeated experiments. These results also confirm that the replication level of ON/C-5B//KE/(V85I) is higher than that of ON/C-5B/KE, ON/C-5B/KE/(K124E) and ON/C-5B/KE/(V85I&K124E).

As the efficiency of colony formation with full-length replicon (ON/C-5B/KE; Fig. 1a) was quite low, we investigated colony formation with subgenomic replicon, ON/3-5B/KE (Fig. 1c). Figure 3 shows the representative result of three independent colony formation assays. The efficiency of colony formation of ON/3-5B/KE was better than that of full-length replicon and the colony formation of in vitro transcript of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 39 (61), 157 (132), 44 (54), and 134 (107), respectively (the numbers in parentheses show another set of result). The efficiency of colony formation of ON/3-5B//KE/(V85I) was greater than that of ON/C-5B/KE and it showed a similar result with that obtained from genome-length replicon.

Inhibition of HCV RNA Replication by IFN and RBV

We examined the inhibitory effect of IFN and RBV on the replication of OR/3-5B/KE. In this experiment, the subgenomic replicon system was used. OR6c cells were treated with IFN at concentrations of 1–20 μM (Fig. 4) and RBV at concentrations of 50, 100, and 200 μM (Fig. 5) after transfection of OR/3-5B/KE derived RNA. Since it is important to know how IFN and RBV treatment is toxic to the cells, we examined cell viability after treatment with 50 and 100 μM of RBV or 2 and 4 units/ml of IFN. The cell viability of OR6c was not

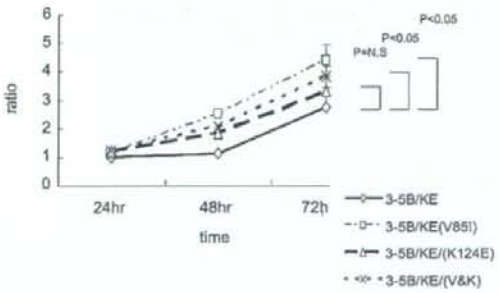

Fig. 2. Effect of amino acid substitutions in NS5B on transient replication activity of replicon. The replication activity of reporter subgenomic HCV replicon with mutation of V85I, K124E, or V85I and K124E (V&K) was compared with that of wild-type in OR6c cells (transient transfection). After 72 hr of transfection, the Renilla luciferase (RL) assay was performed as described in the Materials and Methods section. The relative RL activity (ratio) of mutants was calculated in comparison to that of subgenomic replicon of wild-type (assigned as 1). The data indicate means ± SD of triplicates from three independent experiments. 3-5B/KE: OR/3-5B/KE, 3-5B/KE(V85I): OR/3-5B(K124E), 3-5B/KE/(K124E): OR3-5B/KE/(K124E), 3-5B/KE/(V&K): OR/3-5B/KE/(V85I&K124E).

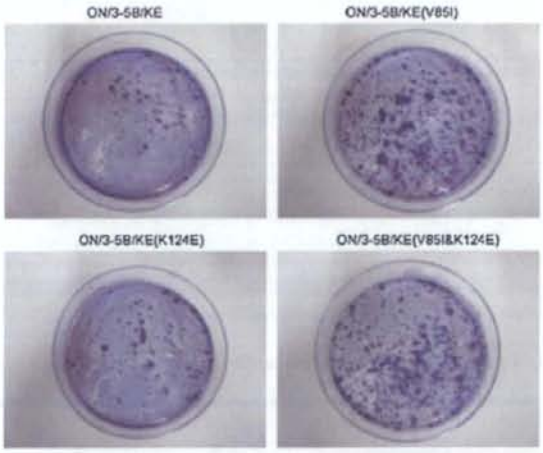

Fig. 3. Colony formation assay of OR6c cells transfected with wild-type and three different mutant replicons. A representative result of colony formation assay using subgenomic replicon RNA (ON/3-5B/KE) system. The efficacy of colony formation was much higher than that of full-length replicon RNA (ON/C-5B/KE). In this series of photographs, colony forming unit of ON/3-5B/KE, ON/3-5B//KE/(V85I), ON/3-5B/KE/(K124E) and ON/3-5B/KE/(V85I&K124E) was 2.7/μg, 6.7/μg, 3.0/μg and 5.4/μg, respectively.

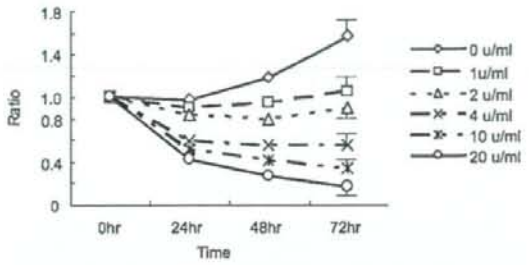

Fig. 4. Dose-dependent inhibition of replication by interferon-α (IFN). OR6 cells were transfected with wild-type replicon (OR/3-5B/KE). Inhibition of HCV RNA replication in the OR6c cell treated with IFN-α was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The cells were treated with IFN-α (0, 1, 2, 4, 10, and 20 u/ml), and the Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

changed by these treatments (Fig. 6), indicating that both IFN and RBV were not toxic to the cells at the indicated concentrations. As shown in Figures 4 and 5, the inhibition of HCV RNA replication occurred in a dose-dependent manner with IFN or RBV treatments. RBV at a concentration of 100 μM inhibited replication of RNA (Fig. 5), but was not toxic to OR6c cells (Fig. 6).

The inhibitory effect of 100 μM RBV on RNA replication in each mutant was also examined. Various biological effect of IFN has been investigated and its effect on cell cycle or cell-differentiation is strong, and we focused on the effect of mutants on RBV treatment. To see this effect, we compared between IFN alone and IFN + RBV. As shown in Figure 7, no difference between three mutants was seen in the treatment with 1 unit/ml of IFN. The proliferation of each mutant RNA was similarly reduced to around a ratio of 0.6. On the other hand, addition of 100 μM of RBV was differently affected by each mutation pattern (Fig. 8). The single mutant with V85I and double mutants with V85I and K124E

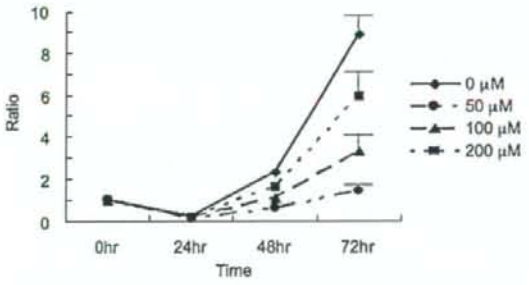

Fig. 5. Dose-dependent inhibition of HCV RNA replication by ribavirin. OR6 cells were transfected with wild-type replicon (OR/3-5B/KE) and treated with ribavirin at concentrations of 50, 100, and 200 μM for 72 hr. Inhibition of HCV RNA replication in the OR6c cell treated with ribavirin (RBV) was shown at the indicated time (24, 48, and 72 hr) from the start of treatment. The relative luciferase activity (%) calculated at each point, where the luciferase activity of non-treated cells at 0 hr was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

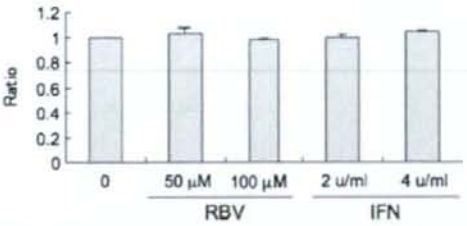

Fig. 6. Cytotoxicity of ribavirin (RBV) or interferon-α (IFN) on replicon RNA in OR6c cells. OR6c cells with OR/3-5B/KE RNA were cultured in the absence or presence of RBV or IFN (50 and 100 μM or 2 and 4 u/ml) for 72 hr, and then the cell viability was determined as described in Materials and Methods Section. The relative cell viability (%) calculated at each point, when viability of non-treated cells was assigned to be 100%, is presented. The data indicate means ± SD of triplicates from three independent experiments.

were significantly increased in RNA proliferation. The degree of inhibition by RBV in OR/3-5B/KE(V85I) and OR/3-5B/KE(V85I&K124E) was significantly lower than that in OR/3-5B/KE, although the difference of OR/3-5B/KE(K124E) was not significant.

Indirect Immunofluorescence

To confirm the presence of replicating full-length RNAs in cells selected for G418 resistance following transfection with ON/C-5B/KE(V85I), one G418-resistant cell colony was selected at random and clonally cultured. We confirmed HCV protein expression by indirect immunofluorescence imaging and observed core protein in the replicon cells (OR6) (Fig. 9c), HCV core protein was demonstrated in the clonally isolated cell line selected after transfection with ON/C-5B/KE(V85I)

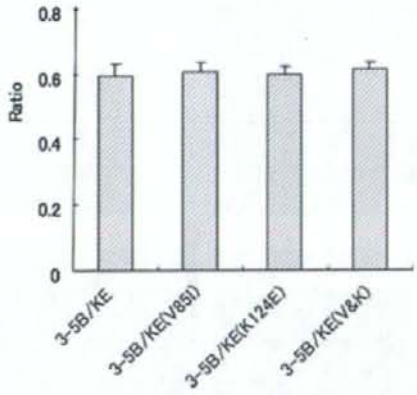

Fig. 7. Effect of interferon-α (IFN) on the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 0 u/ml or IFN 1 u/ml for 72 hr. Renilla luciferase assay was performed as described in Materials and Methods Section. The relative luciferase unit with IFN (1 u/ml) treatment were calculated, where the luciferase unit without IFN treatment was assigned to be 1, and compared between wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from two independent experiments.

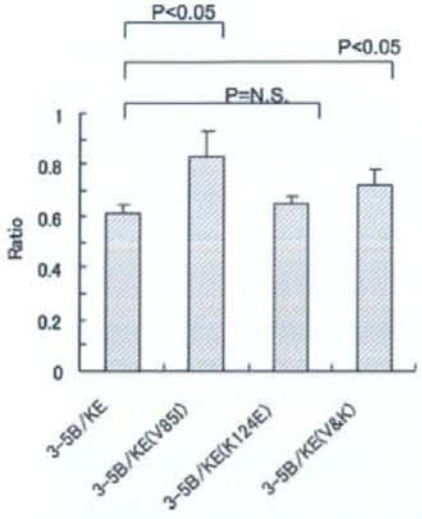

Fig. 8. Effect of interferon-α (IFN) and ribavirin (RBV) combination treatment on the replication levels of the subgenomic HCV replicon possessing the Renilla luciferase reporter. The replication levels of the subgenomic HCV replicons were monitored by luciferase reporter assay with IFN 1 u/ml or IFN 1 u/ml and ribavirin 100 μM for 72 hr. The relative luciferase unit of IFN 1 u/ml and ribavirin 100 mM treatment were calculated, where the luciferase unit of IFN 1 u/ml treatment was assigned to be 1, and compared in wild type (OR/3-5B/KE) and other three mutants (OR/3-5B/KE(V85I), OR/3-5B/KE(K124E), OR/3-5B/KE(V&K)). The data indicate means ± SD of triplicates from three independent experiments.

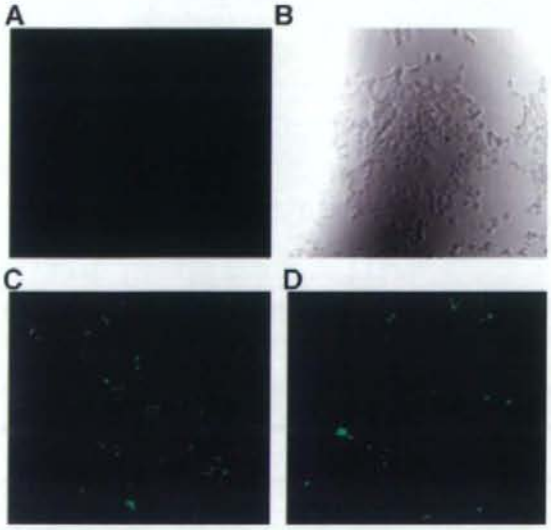

Fig. 9. Indirect immunofluorescence detection of HCV core antigen in normal OR6c cells (cured cell) (**a**), OR6 cells (wild-type HCV replicon) (**c**), and a clonally isolated cell line selected following transfection of OR6c cells with ON/3-5B/V85I (cell line 1) (**d**) and the correspondent phase-contrast microscopic photograph of OR6c cells (**b**).

(Fig. 9d), while it was not observed in the cured cell line (OR6C) (Fig. 9a,b).

DISCUSSION

Predictive factors for a sustained viral response (SVR) in IFN monotherapy or combination therapy have been vigorously investigated in prior studies. In addition to several host and viral factors, such as HCV genotypes, baseline viral load, stage of fibrosis, gender, age, and obesity [Saito et al., 2000, 2006], disappearance of serum HCV RNA during the early phase of therapy or a rapid decrease in HCV RNA levels are significant factors for achieving a SVR [Ferenci et al., 2001]. In our previous study, two distinct amino acid substitutions in the NS5B region of the HCV genome correlated with early viral responses in combination therapy [Kumagai et al., 2004]. NS5B of the HCV genome codes for RdRP, which regulates viral replication. Thus, the detected mutations might increase replication efficacy of HCV or induce resistance to the anti-viral effect of RBV, which could lead to resistance to therapy in the early phase. It was thought that the HCV replicon system would be a good tool for examining the correlation between viral mutation and replication capability. One of the mutation-introduced replicons (V85I) showed a higher replication activity than that of the wild type, and, consistent with our previous clinical study, this mutant was resistant to in vitro RBV treatment. The present study is the first to examine the precise relationship between such mutations and clinical data on the early clearance of HCV during IFN and RBV combination therapy. The mutations of V85I and K124E in NS5B have never been reported in the replicon system.

We investigated the effect of both IFN and RBV on the wild type and three mutants in NS5B at non-toxic concentration to the host cell (Fig. 6). One unit of IFN did not affect the replication of mutants (Fig. 7) but RBV significantly affected the replication of three mutants in the presence of IFN (Fig. 8). These results indicated that the polymorphism of NS5B affect sensitivity to RBV treatment. Although it has been known in the clinical setting that HCV RNA levels are not changed in patients with chronic hepatitis C during RBV monotherapy, our in vitro results showed the reduction of HCV RNA replication with RBV treatment. It was reported that serum levels of RBV in patients with chronic hepatitis C under IFN + RBV combination therapy was very low such as 10^{-14} mM [Naka et al., 2005], however, we can examine the anti-viral effect of much higher levels of RBV on the replicon system without a direct toxic effect of RBV in HuH7 cells. The possibility of a difference between circulating HCV particles and the replicon system in terms of RBV sensitivity may still exist, but this question will be further investigated using a recently developed cell culture system.

We used a dicistronic genome length and subgenome HCV RNA replication systems, which were established previously using HCV RNA from HCV-O infected in non-neoplastic human hepatocyte PH5CH8 cells. For the

cells into which genome-length and subgenomic HCV RNA were introduced, we chose the cloned cell line OR6c, prepared by IFN treatment from subgenomic HCV replicon-supporting cells, since OR6c had a higher efficiency of colony formation (ECF) than its parental HuH-7 cell line in a study of subgenomic HCV replicons [Blight et al., 2002]. It is known that the efficiency of colony formation is unstable, so that the luciferase activity and the colony-forming unit are always discrepant. The impact of ON/C-5B/KE(V85I) on colony formation was about 4 times that of the wild-type replicon in genome length and subgenomic RNAs, and the V85I mutation in NS5B showed 1.5 times higher replication activity in luciferase assay than the wild type in the subgenomic replicon system. Young et al. reported an RBV-resistant NS5B mutation during RBV monotherapy [Young et al., 2003], but this phenylalanine to tyrosine amino acid substitution located at the 415th position in NS5B differed from our amino acid substitution. Replicon cells were selected after G418 exposure, and the replication may be amplified by this selection culture. We sequenced the NS5B region, which includes the 85th and 124th nucleotide portions, from some clones 2 months after G418 selection culture, and we did not found significant mutations. From the present in vitro study and previous clinical study, it may be concluded that at least V85I mutation in NS5B increases viral replication that may cause resistance to RBV treatment.

Two of the patients in the clinical study [Kumagai et al., 2004] had previously been treated with IFN-α monotherapy in our previous study: one patient (Pt 3) has V85 and K124 in the HCV RdRP and the other (Pt 7) had I85 and E124. The former was a good responder to IFN-α and RBV combination therapy, but the latter was not. This result indicated that I85V and E124K substitutions did not affect the response to IFN-α monotherapy, because both types had failed to respond previously to IFN monotherapy. Therefore, we surmised that this amino acid substitution influenced the response to RBV anti-viral activity, which prompted us to examine the effect of RBV on viral replication. Several mechanisms of anti-viral activity of RBV have been proposed [Tam et al., 1999; Maag et al., 2001; Lau et al., 2002], but it is unclear why only the V85I single amino acid substitution induced replication better than the wild type. As shown previously [Kumagai et al., 2004], the 85th amino acid of HCV RdRP is distant from the active site of polymerase but is located near the RNA primer binding site, and this substitution may influence nucleotide misincorporation during polymerization. This 85th position is more important than the 124th position for replication of HCV-O.

This study is the first to examine whether NS5B polymorphism affects the replication efficiency and anti-HCV effect of RBV in an HCV RNA replicon system. It will be interesting to know whether these mutations in other genotypes (genotypes 2 and 3) replicate more efficiently and are more resistant than genotype 1b to RBV alone. Our data suggested that during clinical use of RBV, several mutations in the HCV genome might occur, such as in the isoleucine residue at the 85th position of HCV NS5B, which then affect viral replication and RBV resistance. This viral mutation may be one of the reasons for the failure in early viral clearance by IFN and RBV. There are, however, many factors that influence the success of IFN and RBV combination therapy. The resistance or sensitivity to IFN or peg-IFN, not to RBV, might also affect the early viral response, and many factors in both viral and host sides are known to affect IFN responsiveness, such as NS5A mutations [Enomoto et al., 1996], immunological status [Saito et al., 2000], or irf-1 gene promoter polymorphisms [Saito et al., 2002, 2005]. Together, these factors might determine the efficacy of anti-viral therapy in vivo, and the present in vitro data provides evidence partially supporting our clinical observations that NS5B polymorphisms are associated with early viral clearance during IFN and RBV therapy. However, it is unclear whether this single mutation occurs with peg-IFN plus RBV combination therapy and further studies are necessary. Nevertheless, our report is useful for modeling targets for antiviral compounds for the treatment of HCV.

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Response to Interferon Therapy Affects Risk Factors for Postoperative Recur of Hepatitis C Virus-Related Hepatocellular Carcinoma

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Background: Interferon therapy might reduce recurrence after resection of hepatitis C virus-related hepatocellular carcinoma, especia sustained virologic or biochemical responders.
Methods: Of 209 patients who underwent curative resection for early-stage hepatitis C virus related hepatocellular carcinoma, 7 underwent interferon therapy. A sustained virologic or biochemical response was achieved in 40 patients (SVR/BR group). Thirty no r and 139 patients who had not received interferon therapy were classified as the NR/non-IFN group. Risk factors for postoperative rec each group were analyzed.
Results: The tumor-free survival rates in the SVR/BR group were significantly higher than those in the NR/non-IFN group. By m analysis, the presence of multiple tumors was independently associated with recurrence after resection in both groups, while histologic of cirrhosis was another independent risk factor for postoperative recurrence in the NR/non-IFN group.
Conclusions: Newly multicentric carcinogenesis after resection could be suppressed when active hepatitis is controlled by interfero Patients with single hepatitis C virus related hepatocellular carcinoma detected after successful interferon therapy are good candidates f resection. Adjuvant interferon therapy might be indicated for patients who undergo curative resection for single hepatocellular c associated with hepatitis C.
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KEY WORDS: hepatocellular carcinoma; hepatitis C virus; interferon; postoperative outcome

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in Asia and Africa, and its incidence is increasing in the Western world [1,2]. Since chronic infection with hepatitis C virus (HCV) is closely associated with development of HCC [3], surveillance of patients with HCV infection has made possible the detection of HCC at an early stage [4]. Patients with early-stage HCC should be considered for any of the available treatment options, including local ablation therapy, liver transplantation, and surgical resection. Recently, radiofrequency ablation (RFA) has been accepted as new technique for the treatment of HCC. Although several reports have shown that RFA for small HCC can provide effective local control of tumors and favorable survival rates, RFA cannot achieve complete necrosis of tumors in all cases [5,6]. Although the long-term outcomes after liver transplantation are satisfactory in patients with early-stage HCC [7,8], liver transplantation for HCC is limited because of the shortage of donors and the high associated costs [9]. Hepatic resection therefore has been the first-line therapeutic option for early-stage HCC [10]. However, the outcome after hepatic resection for HCC is still unsatisfactory because of the high incidence of tumor recurrence, especially in the liver remnant [11,12].

Persistent active hepatitis and extensive fibrosis play important roles in the development of HCV-related HCC [13–15]. In treating HCC, it is therefore necessary to control recurrences that originate from intrahepatic metastases and also to control newly multicentric carcinogenesis after surgery [16–24]. Previous studies have indicated that HCC is less likely to develop in those in whom interferon (IFN) therapy was effective at normalizing the serum alanine aminotransfer- ase (ALT) activity, even when the HCV RNA did not disappear

patients who have had a curative resection for HCV-related H continuous active hepatitis is strongly associated with recurr curative resection [33–36]. We have reported that patients HCC is detected after IFN therapy are good candidates f resection because of the low incidence of postoperative re [33], especially among virologic or biochemical responder Moreover, postoperative long-term IFN-α therapy appears t the incidence of recurrences after a curative resection of HC HCC [36]. We therefore hypothesized that IFN therapy mig recurrence due to new carcinogenesis after hepatic resection i way that IFN therapy decreases the incidence of HCV-relate

Intrahepatic metastases and multicentric carcinogen resection of HCC might be associated with different ri [16–21]. Tumor factors, such as venous invasion and the p multiple tumors, whose major mechanism is intrahepatic n have been found to be risk factors for early recurrence [On the other hand, several investigators have reported t multicentric carcinogenesis was closely linked to the stat underlying liver [16–21]. In this retrospective study, we in whether different risk factors are associated with the pos recurrence of HCV-related HCC among each group based response to IFN therapy.

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PATIENTS AND METHODS

Patients

Between January 1993 and March 2007, 279 patients with anti-HCV antibody, but not hepatitis B surface antigen, underwent a curative hepatic resection for HCC in the Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Hospital. Curative surgery was defined as the complete removal of all macroscopic tumors. The absence of tumor cells along the parenchymal transection line was confirmed histologically. Four perioperative deaths occurred from postoperative liver failure. Of the 279 patients, exclusive of operative deaths, 209 patients had early-stage HCC who met the Milan criteria [7] (single tumor ≤5 cm, maximum of three total tumors with none >3 cm, no major vessel invasion, and no extrahepatic involvement) were included in this study. Forty-three patients underwent a hepatic resection for HCC that had been detected after IFN therapy, and 27 patients received IFN therapy after curative resection. The type, dosage, and duration of IFN varied. The response to IFN therapy was determined virologically and biochemically. Of the 70 patients who received IFN therapy, 32 patients obtained a sustained viral response (SVR) that was defined as return of ALT activity to within the reference range and no detectable serum HCV RNA for at least 6 months after IFN therapy. A biochemical response (BR), which was defined as normalized ALT activity for at least 6 months after IFN therapy with or without the transient disappearance of serum HCV RNA, was obtained in eight patients. These 40 patients were defined as the responsive (SVR/BR) group, while the other 30 patients had no decrease in their ALT activity and had persistent serum HCV RNA. The NR/non-IFN group included these 30 no responders and 142 patients who had not received IFN therapy.

All patients were followed from resection until death or the end point of this study (December 31, 2007). The follow-up period ranged from 65 to 4,894 days (median, 1,491 days).

Detection of Recurrence

Serum alpha-fetoprotein was measured 1 month after surgery and every 3 months thereafter. Hepatic ultrasound scanning or CT was performed 1 month after surgery and every 3 months there a recurrence of HCC was strongly suspected on the basis markers or imaging, selective hepatic angiography, ultrasou biopsy, or both were performed to establish a definitive dia

Surgical Procedure and Pathology

The type of operative procedure was described using th 2000 system [37]. A hemihepatectomy, bi-segmentect segmentectomy were all assumed to be anatomic resect classification system of the Liver Cancer Study Group of was used to categorize the histological findings. The grade active hepatitis) and stage (degree of hepatic fibrosis) in cancerous portions were determined by the score of the activity index [39,40], which was determined by four even periportal necrosis with or without bridging necrosis, i degeneration with focal necrosis, portal inflammation, an Two pathologists without any knowledge of the clinical and data examined all materials.

Statistics

Differences in clinicopathologic findings were analyz Mann–Whitney *U*-test or Chi-squared test. The tumor-fre rates were calculated by the Kaplan–Meier method, compared with the log-rank test. Covariates with *P* value the log-rank test were entered into a Cox regression m forward stepwise selection. The variables chosen were ag <65 years), gender, aspartate aminotransferase (AST) activi >40 IU/L), ALT activity (≤45 or >45 IU/L), total biliru or >1.0 mg/dl), albumin concentration (<3.5 or ≥3.5 g/d count (≥10 or <10 $\times 10^4/\mu l$), serum alpha-fetoprotein cor (≤20 or >20 ng/ml), the largest diameter of the main tumo ≥3.0 cm), the degree of differentiation of the main tur moderate or poor), the number of tumors (single or mul presence of portal invasion, the grading score (0–1 or 2–4), t score (0–3 or 4), and type of hepatic resection (anatomic re nonanatomic resection).

TABLE 1. Clinicalpathologic Findings in Patients With Hepatocellular Carcinoma

	SVR/BR (n = 40)	NR/non-IFN (n = 169)	*P*-value
Age (year)	65 (54, 71)	67 (56, 75)	0.010
Gender (M/F)	35:5	140:29	0.473
AST activity (IU/L)	43 (27, 91)	66 (36, 102)	<0.001
ALT activity (IU/L)	43 (26, 139)	74 (32, 117)	0.022
Albumin (g/dl)	4.1 (3.6, 4.4)	3.6 (3.3, 4.1)	<0.001
Total bilirubin (mg/dl)	0.8 (0.5, 1.3)	0.8 (0.5, 1.3)	0.436
Platelet count ($10^4/mm^3$)	14 (9, 21)	13 (8, 21)	0.100
Child-Pugh score (A/B)	39:1	156:13	0.238
High AFP (>20 ng/ml)	15	88	0.096
Anatomic resection	13	57	0.882
Tumor size (cm)	2.0 (1.5, 3.8)	2.5 (1.5, 4.0)	0.082
Number of tumors			
Single/multiple	27:13	132:37	0.157
Portal invasion	8	33	0.946
TMN stage			
I/II/III	7:21:12	27:101:41	0.684
Tumor differentiation			
Well/moderate or poor	4:36	18:151	0.904
Histologic activity index score			
Grade 0–1/2–4	9:31	34:135	0.738
Stage 0–3/4 (cirrhosis)	28:12	82:87	0.014

RESULTS

The clinical features, laboratory test results, and pathologic findings of the surgical specimens are summarized in Table I. The mean age in the SVR/BR group was significantly younger than those in the NR/non-IFN group. Although the serum concentration of albumin just before surgery was significantly higher in the SVR/BR group than in the NR/non-IFN group, there were no differences in the serum concentrations of total bilirubin, the platelet counts, and the proportion of Child-Pugh A cirrhosis between two groups. The serum activities of aspartate aminotransferase and ALT just before surgery were significantly lower in the SVR/BR group than in the NR/non-IFN group. Although there were no differences in tumor-related factors, the proportion of stage 4 fibrosis (cirrhosis) in the SVR/BR group was lower than that in the NR/non-IFN group.

The tumor-free survival rates for the patients in the SVR/BR group and the NR/non-IFN group were 71% and 46% at 3 years; and 54% and 23% at 5 years, respectively (Fig. 1). The recurrence rates in the NR/non-INF group steadily increased over time. Conversely, only one patient in the SVR/BR group had a recurrence of HCC more than 4 years after resection. The tumor-free survival rate was higher in the SVR/BR group than in the NR/non-INF group ($P < 0.001$).

Table II shows the univariate analysis of risk factors for postoperative recurrence in each group according to the response to IFN therapy. In the SVR/BR group, the low platelet count, the presence of multiple tumors and a nonanatomic resection were possible risk factors for recurrence after resection. In the NR/non-INF group, low albumin concentration, the presence of multiple tumors, and histologic evidence of cirrhosis (stage 4 fibrosis) were associated with lower tumor-free survival rates. By multivariate analysis, the presence of multiple tumors was an independent risk factor associated with postoperative recurrence in both groups (Table III). In addition, histologic evidence of cirrhosis was another independent predictor of postoperative recurrence in the NR/non-INF group.

DISCUSSION

Because of recent progress in surgical techniques and perioperative management, the perioperative outcome of hepatic resection for

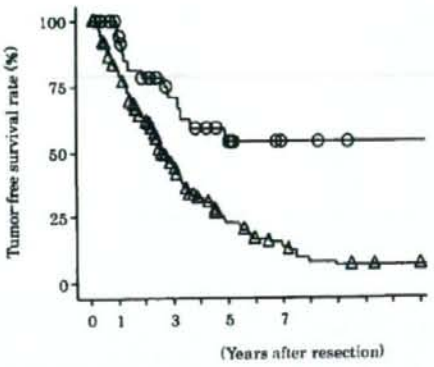

Fig. 1. Tumor-free survival rates after curative resection c cellular carcinoma. Open circles: SVR/BR group (n = 4 triangles: NR/non-IFN group (n = 169).

HCC has markedly improved [10]. However, the high inc recurrence after resection remains a serious problem in pati HCV-related HCC [11,12]. Postoperative recurrence of HC HCC is thought to result from the growth of intrahepatic met the residual liver and metachronous, multicentric carcinogene the underlying liver disease [16–24]. Accordingly, different may be needed for the prevention and management of t patterns of HCC recurrence.

Cirrhosis is a major risk factor for the development of HC HCC [25,26]. In patients with chronic active hepatitis, fibro liver is established through repetitive necroinflammation a eration. It has been reported that the risk for the developmen increases with the degree of liver fibrosis, from 0.5% among with stage 0 or 1 fibrosis to 7.9% among patients with stage (cirrhosis) [25]. Several investigators have reported that l

TABLE II. Univariate Analysis for Factors Associated With Postoperative Recurrence

Factors	Number of patients	Tumor-free survival rate (95% CI) 3 years (%)	5 years (%)	P-value
SVR/BR group				
Type of resection				
Anatomic	13	90 (71–100)	90 (71–100)	0.032
Limited	27	62 (42–82)	39 (16–61)	
Number of tumors				
Single	27	81 (64–98)	68 (45–90)	0.001
Multiple	13	49 (16–81)	24 (0–53)	
Platelet count (10^4/mm^3)				
≥10.0	35	81 (66–96)	61 (40–81)	0.010
<10.0	5	20 (0–55)	20 (0–55)v	
NR/non-IFN group				
Albumin (g/dl)				
≥3.5	113	52 (42–62)	26 (16–35)	0.021
<3.5	56	33 (20–47)	17 (5–30)	
Number of tumors				
Single	132	49 (40–58)	26 (17–35)	0.007
Multiple	37	36 (20–52)	11 (0–25)	
Stage of fibrosis				
0–3	82	57 (45–68)	34 (22–46)	0.003

TABLE III. Multivariate Analysis of Factors Predicting Postoperative Recurrence

	Multivariate risk ratio*	*P*-value
SVR/BR group		
Presence of multiple tumors	3.9 (1.3–11.8)	0.018
NR/non-IFN group		
Presence of multiple tumors	1.7 (1.1–2.6)	0.014
Stage 4 fibrosis (Cirrhosis)	1.7 (1.2–2.4)	0.005

*Risk ratio with 95% confidence interval in parentheses.

status of the underlying liver disease, such as evidence of fibrosis and active hepatitis, is also associated with recurrence of HCC after resection, which suggests that newly multicentric carcinogenesis could play an important role in the development of recurrence after resection [16–23]. The incidence of newly multicentric carcinogenesis is considerably high, which was associated with approximately 50% of postoperative recurrences [24]. Several investigators have identified that IFN therapy suppresses the development of HCC by causing active hepatitis to go into remission and by improving hepatic fibrosis, which increases the survival rate in patients with HCV infection [25–32]. Moreover, SVR/BR to IFN therapy was identified as an independent factor for a lower incidence of postoperative recurrence [34,35]. In this study, the tumor-free survival rate was higher in the SVR/BR group than in the NR/non-INF group. The majority of HCC recurrence was detected in the SVR/BR group within 4 years after surgery, while recurrence of HCC occurred throughout the follow-up period in the NR/non-INF group. Histologic evidence of cirrhosis was a risk factor for postoperative recurrence in the NR/non-IFN group but not in the SVR/BR group. These findings support the hypothesis that controlling active hepatitis by IFN therapy can improve hepatic fibrosis and prevent the development of multicentric carcinogenesis after resection.

The growth of micrometastases via vascular invasion is considered another major form of HCC recurrence [24]. Because of the high likelihood of micrometastases of HCC spreading through the portal venous system, anatomic resection is theoretically effective for the eradication of micrometastases [41,42]. In this study, the tumor-free survival rate for patients who underwent an anatomic resection was much higher than that for patients who underwent nonanatomic resection in SVR/BR group. Although some studies have suggested that anatomic resection has a beneficial effect on recurrence-free survival after hepatic resection for single HCC [43,44], the superiority of an anatomic resection has been unclear. Previous studies identified that tumor-related factors such as tumor size, the presence of multiple tumors and portal invasion are significant factors associated with postoperative recurrence of HCC [11,16–18]. In this study, the presence of multiple tumors was an independent risk factor for postoperative recurrence, irrespective of the response to IFN therapy. Therefore, in patients with multiple tumors liver transplantation could be considered because the efficacy of adjuvant therapeutic approaches, such as postoperative hepatic arterial chemotherapy remains controversial. Conversely, curative resection provides the chance of cure for patients with single HCV-related HCC detected after successful IFN therapy. Patients who undergo curative resection for single tumor are good candidates for adjuvant IFN therapy.

CONCLUSIONS

Late recurrence of HCC after resection decreased and the histologic status of the underlying liver disease was not associated with postoperative recurrence in the SVR/BR group. These results suggest that newly multicentric carcinogenesis after resection could be

However, there was a high incidence of postoperative in patients with multiple tumors irrespective of their re IFN therapy. Patients with single HCV-related HCC det successful IFN therapy are good candidates for surgical Adjuvant IFN therapy might be indicated for patients wh curative resection for single HCV-related HCC.

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CLINICAL STUDIES

Differences in molecular alterations of hepatocellular carcinoma between patients with a sustained virological response and those with hepatitis C virus infection

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Abstract

Background/Aims: The mechanism of hepatocarcinogenesis remains unclear in patients in whom hepatitis C virus (HCV) disappears after interferon (IFN) therapy. We compared molecular alterations in hepatocellular carcinoma (HCC) between patients with a sustained virological response (SVR) to IFN and patients with HCV. *Methods:* The study group comprised 44 patients with HCV and 13 patients with SVR. One patient in the SVR group had two tumour nodules, both of which were examined. Mitochondrial DNA (mtDNA) mutations in displacement-loop lesions were directly sequenced. Mutation of the TP53 gene was examined by direct sequencing. The methylation status of *p16*, *p15*, *p14*, *RB* and *PTEN* genes was evaluated by a methylation-specific polymerase chain reaction. *Results:* The average number of mtDNA mutations was 4.2 in 44 HCCs with HCV and 2.0 in 14 HCCs with SVR ($P = 0.0021$). mtDNA mutation was less frequently detected in HCCs from patients with SVR than in patients with HCV. TP53 mutations were detected in 12 (27%) of 44 HCCs with HCV and 2 (14%) of 14 SVR-HCCs. Hypermethylation of the *p16*, *p15*, *p14*, *RB* and *PTEN* promoters was, respectively, detected in 34, 13, 8, 12 and 11 of 44 HCCs from patients with HCV and 14, 0, 0, 2 and 2 of 14 HCCs from patients with SVR ($P = 0.049$, 0.021, 0.085, 0.322 and 0.402). Hypermethylation of *p16* was one of the most important alterations in SVR-HCC. *Conclusions:* Molecular alterations in hepatocarcinogenesis of patients with SVR-HCC were different from those of patients with continuous HCV infection.

Hepatitis C virus (HCV) is one of the most important risk factors for hepatocellular carcinoma (HCC). Clinical studies have suggested that HCV induces inflammation in the liver, followed by the accumulation of reactive oxygen species (ROS), which promote mutations in the human genome (1, 2). Persistent inflammation also results in repeated hepatocyte death and regeneration, leading to the gradual accumulation of DNA mutations in hepatocytes. Point mutations in tumour suppressor genes, including TP53, have been confirmed in hepatic cirrhosis in patients with HCV (3). Epigenetic alterations, such as methylation of the promoter of cell cycle gene inhibitors with the resulting loss of its expression, have been frequently detected in liver cirrhosis with viral infection (4, 5). Continuous inflammation induces genetic or epigenetic alterations, or both, in hepatocytes, culminating in a preneoplastic condition. HCV itself is an oncogenic virus. HCV core protein or HCV NS5A protein has oncogenic potential function in animal models without inflammation (6, 7). *In vitro* studies have suggested that HCV protein modifies host immunity to sustain infection (8). The suppression of immunological response is attributed to the failure to eliminate neoplastic cells from the liver. These findings suggest that cooperation between virus-induced chronic inflammation and HCV coding proteins accelerates carcinogenesis in the liver.

Interferon (IFN) has potent antiviral activity against HCV. Antiviral therapy with pegylated IFN in combination with ribavirin produces a sustained virological response (SVR) in approximately 60% of patients with chronic hepatitis C (9, 10). Complete eradication of HCV by antiviral therapy is associated with a considerable reduction in the incidence of HCC (11, 12). Nevertheless, recent studies have shown that HCC develops in 2.5–4.2% of patients after eradication of HCV by IFN therapy (13–15). It is therefore important to delineate important features of HCC that develop after the elimination of HCV as compared with those established during sustained HCV infection. Makiyama *et al.* (15) speculated that cancer cells already exist in the liver before HCV eradication by IFN treatment. The integration of HBV DNA because of past HBV infections (16) or occult HCV infections (17) may be linked to SVR-HCC. However, the molecular mechanism leading to the development of SVR-HCC remains obscure.

In the present study, we compared genetic alterations in surgically resected specimens of HCCs between patients with SVR and those with continuous HCV infection. Our results might contribute to a better understanding of the molecular changes in

the liver of patients in whom HCC develops after the eradication of HCV.

Patients and methods

Patients

Thirteen consecutive patients who underwent surgical resection of HCC in Osaka City University Hospital after eradication of HCV by IFN monotherapy from 1998 June through 2007 July (SVR group) were studied (Table 1). One patient in the SVR group had two tumour nodules, both of which were examined. As a control, 44 HCV-RNA-positive patients with HCC were studied. Thus, 58 HCC samples and 57 noncancerous tissue samples were evaluated. One portion of each sample was frozen in liquid nitrogen immediately after resection and stored at − 80 °C until analysis. Total RNA and DNA were extracted from these portions by conventional methods as described previously (18). None of the patients had a history of exposure to aflatoxin B1, more than 30 g/day of alcohol intake, insulin administration, hereditary haemochromatosis or other liver diseases such as hepatitis B, autoimmune hepatitis and primary biliary cirrhosis. The activity of hepatitis and stage of fibrosis were determined according to a modified version of Desmet's classification in liver tissue specimens before IFN therapy and in noncancerous liver tissue obtained intra-operatively (19).

Sequencing the displacement loop region of mitochondrial DNA

Each DNA sample (50 ng) was subjected to amplification by polymerase chain reaction (PCR) with the use of overlapping sets of primers to screen the entire mitochondrial genome. To avoid coamplification of nuclear pseudogenes, the primers were selected with the use of mitochondrial DNA (mtDNA)-depleted cells established as described previously (2, 20). PCR (an initial incubation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) was performed in a final volume of 50 μl with a GeneAmp PCR system 9600 (Perkin-Elmer Life Sciences Japan, Tokyo, Japan). Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer Life Sciences Japan) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (*Applied Biosystems*, Tokyo, Japan). The sequence of the displacement (D)-loop (nucleotides 100–600) was examined for all 57 patients with HCC. All mutations were confirmed by repeated DNA sequencing.

Direct sequencing for TP53

We directly sequenced exons 5 through 8 of TP53 genes, in which 98% of TP53 mutations are detected (21), in 58 tumours. One hundred nanograms of genomic DNA was subjected to 35 PCR cycles (94, 55 and 72 °C for 0.5, 0.5 and 1 min respectively) with rTaq DNA polymerase (TakaraBio Co. Ltd, Otsu, Japan). After the PCR products were purified with a QIAquick PCR purification kit, we sequenced the amplified products with a DNA sequencing system and a Dye Terminator Cycle Sequencing FS Ready Reaction kit.

Methylation-specific polymerase chain reaction

Bisulphite modification of genomic DNA was performed as described by Herman *et al.* (22). Briefly, 1 μg of DNA was denatured with NaOH, and 10 mM hydroquinone and 3 M sodium bisulphite were successively added to the mixture. The sample was incubated at 50 °C for 16 h. Modified DNA was purified with the use of Wizard DNA purification resin (Promega Corporation, Madison, WI, USA), followed by ethanol precipitation. DNA methylation patterns were determined by chemical modification of the unmethylated cytosines to uracil and subsequent PCR, using primers specific for either methylated or modified unmethylated DNA. The primers used in this study are shown in Table 2 (23, 24). The PCR amplification procedure has been described previously (5). Ten microlitres of each PCR product was loaded directly onto nondenaturing 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

Table 1. Clinical characteristics of patients with sustained virological response-hepatocellular carcinoma and hepatitis C virus-hepatocellular carcinoma

	SVR-HCC	HCV-HCC	*P*-value
n	13	44	
Male/female	13/0	44/0	
Age	64.3 (55–73)	64.0 (34–79)	0.977
Anti-HCV(+)/HCV-RNA(+)	13/0	44/44	
HBs antigen positivity	0	0	
IFN therapy	13	0	
ALT (IU/L)	35.0 (17–81)	73.2 (13–188)	0.0001
Diabetes mellitus With/without/unknown	2/11/0	13/28/3	0.25
Alcohol habits Positive/negative/unknown	5/8/0	23/17/4	0.23
Tumour differentiation Well/moderately/poorly	0/4/10	5/20/19	0.066
Noncancerous liver Cirrhosis/noncirrhosis	4/9	20/24	0.34
Tumour diameter (mm) (average)	43.1 (12–125)	38.3 (10–180)	0.756
Extrahepatic metastasis	0	0	

ALT, alanine aminotransferase; HBs antigen, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological reaction.

Semiquantitative reverse-transcription polymerase chain reaction analysis

To investigate *p16* mRNA expression, we performed reverse-transcription PCR (RT-PCR) with total RNA from 35 tumours and 27 noncancerous lesions. Briefly, 1 μg of the RNA was used as a template to generate complementary DNA (cDNA) using random hexamers and reverse transcriptase. The cDNA was used for PCR amplification. Primer sequences were 5′-CCACCCCGC TTTCGTAGTTTT-3′ (upper primer) and 5′-TGCGAGGCTCG CAAGAAAT-3′ (lower primer) for *p16* and 5′-CCTCGCCTT TGCCGATCC-3′ (upper primer) and 5′-GGATCTTCATGAGG TAGTCAGTC-3′ (lower primer) for β-actin. The PCR procedure for *p16* consisted of one cycle at 95 °C for 12 min, 30 cycles at 95 °C for 30 s, 51 °C for 1 min and 72 °C for 30 s, and one cycle at

Table 2. Primers used for methylation-specific polymerase chain reaction

Gene		Sequence
p16	Unmethylated	5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (sense)
		5′-CAACCCCAAACCACAACCATAA-3′ (antisense)
	Methylated	5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense)
		5′-GACCCCGAACCGCGACCGTAA-3′ (antisense)
p15	Unmethylated	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′ (sense)
		5′-CCATACAATAACCAAACAACCAA-3′ (antisense)
	Methylated	5′-GCGTTCGTATTTTGCGGTT-3′ (sense)
		5′-CGTACAATAACCGAACGACCGA-3′ (antisense)
p14	Unmethylated	5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′ (sense)
		5′-CACAAAAACCCTCACTCACAACAA-3′ (antisense)
	Methylated	5′-GTGTTAAAGGGCGGCGTAGC-3′ (sense)
		5′-AAAACCCTCACTCGCGACGA-3′ (antisense)
RB		5′-CTTTGTATAGCCCCGTTAAGTG-3′ (sense)
		5′-GTCATGAGGAATTAAACTGGGA-3′ (antisense)
PTEN	Unmethylated	5′-GTGTTGGTGGAGGTAGTTGTTT-3′ (sense)
		5′-ACCACTTAACTCTAAACCACAACCA-3′ (antisense)
	Methylated	5′-TTCGTTCGTCGTCGTCGTATTT-3′ (sense)
		5′-GCCGCTTAACTCTAAACCGCAACCG-3′ (antisense)

72 °C for 3 min. That for β-actin consisted of one cycle at 94 °C for 3 min, 24 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s, and one cycle at 72 °C for 3 min. Ten microlitres of each PCR product was loaded directly onto nondenaturing 8% polyacrylamide gels, and the gels were stained with SYBR Greene (BioWhittaker Molecular Applications, Rockland, ME, USA) according to the manufacturer's protocol. The intensity of the bands was quantified by densitometry.

Statistical analysis

Age, tumour size, liver function and mtDNA mutations were compared between the two groups with the Mann–Whitney *U* test. Histological findings, diabetes mellitus, alcohol use, tumour differentiation, TP53 mutation and methylation status were compared between the two groups with the χ^2 test.

Ethical considerations

This study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medical.

Results

Histological findings in patients with sustained virological response

In patients with SVR, the period from the end of IFN treatment to hepatectomy for HCC ranged from 13 to 156 months. Histological examinations, performed in 11 of the 13 patients with SVR-HCCs, showed that the staging of hepatic fibrosis improved in five patients and the grade of hepatic activity improved in eight patients (Table 3).

Mitochondrial DNA mutations of hepatocellular carcinoma

Mutations of mtDNA were found in both HCC and noncancerous liver tissue. Previously, three mutation sites in mtDNA have been reported to be unique for the Japanese. Excluding these sites, we evaluated the average number of mtDNA mutations in D-loop lesions (Table 4). The average number of mtDNA mutations in D-loop lesions was 4.2 in 44 HCCs with HCV and 2.0 in 14 HCCs from SVR patients. The average number of mtDNA mutations in D-loop lesions was 2.8 in 44 noncancerous lesions with HCV and 1.3 in 13 noncancerous lesion from SVR patients. No specific mutation in mtDNA of SVR-HCC was found in the present study. The frequency of mtDNA mutations in HCC was significantly lower in SVR patients than in HCV patients ($P = 0.0021$). The frequency of mtDNA mutations was also lower in noncancerous livers of SVR patients ($P = 0.007$). In the present study, no regularity of mtDNA mutations was found in the D-loop region.

TP53 mutation analysis

TP53 mutations were detected in 12 (27.3%) of 44 HCCs with HCV (Table 4). In detail, TP53 was mutated in codon 123, TAT to TTC; codon 132, AAG to TTG; codon 133, ATG to TTG; codon 158, CGC to CTC; codon 189, GCC to GTC; codon 220, TAT to TGT; codon 246, ATG to GTG; codon 272, GAG to GTG; codon 275, TGT to TAT; and codon 271, CAT to CGT. Two cases were mutated by insertion in exons 5 and 8. The histological findings showed that HCCs with TP53 mutations consisted of seven moderately differentiated and five poorly differentiated HCCs. TP53 mutations were detected in two (14.3%) of 14 HCCs from the 13 patients in whom HCV was eradicated by IFN therapy. In detail, TP53 was mutated in codon 135, TGC to TGG and codon 242, TGC to TTC. The histological findings showed that HCCs with TP53 mutations in SVR patients consisted of two poorly differentiated HCCs.

Methylation pattern of hepatocellular carcinoma

In patients with HCV, hypermethylation of *p16*, *p15*, *p14*, *RB* and the *PTEN* promoter was, respectively, detected in 34 (77.3%), 13 (29.5%), 8 (18.2%), 12 (27.3%) and 11 (25.0%) of 44 HCCs and 13 (29.5%), 14 (31.8%), 4 (9.1%), 11 (25.0%) and 5 (11.4%) of 44 noncancerous liver samples (Fig. 1A). In patients with SVR, hypermethylation of *p16*, *p15*, *p14*, *RB* and the *PTEN* promoter was, respectively, detected in 11 (100%), 0 (0%), 0 (0%), 2 (14.3%) and 2 (14.3%) of 14 HCCs and 2 (15.4%), 0 (0%), 0 (0%), 2 (15.4%) and 0 (0%) of 13

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Table 3. Clinical course of patients with sustained virological response-hepatocellular carcinoma

Case	Pre-IFN therapy				Span for carcinogenesis after IFN therapy (months)	At operation		
	Genotype	HCV-RNA	*F* factor	*A* factor		*F* factor	*A* factor	BMI
56	1b	1 MEq	2	2	45	2	1	23.7
101	2a	+	3	2	19	4	2	23.7
149	2a	1.1 MEq	4	3	20	4	2	23.6
196	2b	+	2	2	41	1	2	23.4
198	2a	0.4 MEq	2	2	103	1	1	21.5
200	2a	1.1 MEq	2	2	13	2	1	24.6
221	2a	0.9 MEq	2	3	80	2	2	18.1
268	Unknown	+	Unknown	Unknown	144	1	1	20.3
269	2a	0.4 MEq	2	3	156	0	0	23.6
271	1b	+	4	1	156	3	1	28.1
325	1b	300 KIU	3	2	15	2	1	25.2
327	2b	150 KIU	3	3	36	4	2	26.8
328	1b	+	Unknown	Unknown	14	4	2	27.6

BMI, body mass index; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response

Table 4. Comparison of mutation in the displacement-loop of mitochondrial DNA, mutation in TP53 and methylation between sustained virological response-hepatocellular carcinoma and hepatitis C virus-hepatocellular carcinoma

	SVR-HCC	HCV-HCC	*P*-value
Mean mutation number in D-loop of mtDNA	2.0	4.2	0.0021
TP53 mutation	14.3%	27.3%	0.322
Methylation			
p16	100.0%	77.3%	0.049
p15	0.0%	29.5%	0.021
p14	0.0%	18.2%	0.085
RB	14.3%	27.3%	0.322
PTEN	14.3%	25.0%	0.402

D-loop, displacement-loop; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; mtDNA, mitochondrial DNA; SVR, sustained virological response

noncancerous liver samples (Fig. 1B). Methylation of *p14*, *p15*, *RB* and *PTEN* was thus slightly but not significantly more frequent in HCV-HCC than in SVR-HCC.

Expression of *p16* mRNA in hepatocellular carcinoma

Expression of *p16* mRNA was examined in 29 patients with HCV-HCC and six with SVR-HCC. In six SVR-HCCs with *p16* promoter methylation, *p16* mRNA expression was lower than that in the noncancerous liver (Fig. 2). In 29 HCV-HCCs with *p16* methylation, *p16* mRNA expression was lower than that in HCC without *p16* methylation.

Discussion

In agreement with previous studies, all patients with SVR-HCC were males in the present study (15), suggesting that sex-related factors have a role in SVR-HCC. We, therefore, studied male patients with HCV-HCC and matched subjects with SVR-HCC. First of all, mtDNA mutations were frequent in HCC as well as in noncancerous liver tissues from patients with HCV (2, 25). Chronic viral inflammation induces ROS production, followed by mtDNA damage in the liver, which is speculated to contribute to hepatocarcinogenesis (25). In contrast to mtDNA mutations, the frequency of mtDNA mutations was low in SVR liver. Histological examination of noncancerous liver tissue showed that persistent inflammation was minimal or absent in SVR patients. Nishikawa *et al.* reported that IFN therapy reduces the frequency of mtDNA mutations in the liver of patients with chronic hepatitis C. In their study, a reduced frequency of mtDNA mutations was detected only in patients whose transaminases were normalized by IFN therapy in association with HCV elimination (26). Our study also showed that the frequency of mtDNA mutations was reduced in the liver of SVR patients. In the present study, no patient with HCV-HCC received IFN. Therefore, we could not exactly clarify which factor was more closely related to fewer mtDNA mutations in SVR-HCC, IFN or HCV eradication. However, we speculate that chronic inflammation was not related to the development of HCC in SVR patients.

Destruction of tumour suppressor gene function is thought to be a critical step in carcinogenesis. Previous studies showed that TP53 mutations were detected in 27% (21) and 38.3% (27) of HCCs with viral infection. These high rates were apparently related to the late stage of hepatocarcinogenesis. In agreement with these previous reports, TP53 was mutated in seven moderately differentiated HCC and five poorly differentiated HCC (27.7%) in the 44 patients with HCV in our study. To our knowledge, no previous study has reported TP53 mutations in SVR-HCC. We found two TP53 mutations in 14 SVR-HCC, including dedifferentiated lesions. mtDNA damage induced by chronic viral hepatitis correlates with genomic injury. It was speculated that a decrease in mtDNA mutations followed loss of TP53 mutations. Although the small number of the SVR-HCCs examined in our study precludes firm conclusions, TP53 alterations might differ between SVR-HCC and HCV-HCC.

Next, we showed epigenetic alterations in both HCV-HCC and SVR-HCC. Previous studies have reported that *p16*, *p15*,

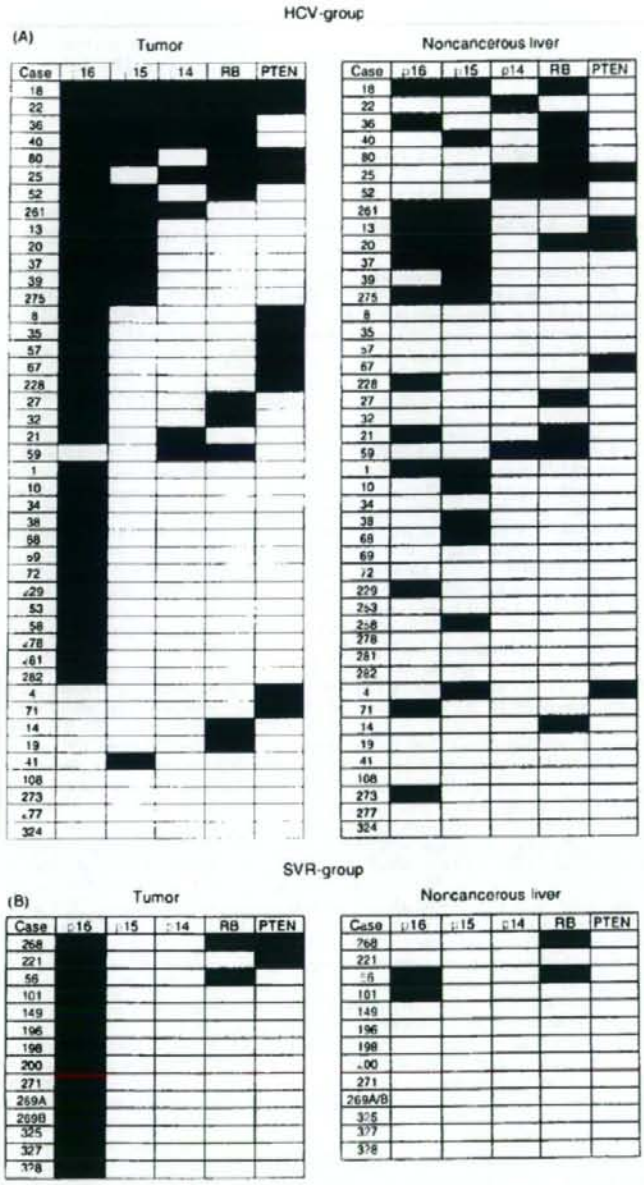

Fig. 1. Methylation patterns of *p16*, *p15*, *p14*, *RB* and *PTEN* promoters in 44 hepatocellular carcinomas (HCC) and 44 noncancerous liver samples from the hepatitis C virus (HCV) group were examined by methylation-specific polymerase chain reaction (MSP) (A). Methylation patterns were also examined by MSP for 14 HCCs and 13 noncancerous liver samples from the sustained virological response (SVR) group (B). Black boxes indicate methylated sequences, whereas blank boxes indicate unmethylated sequences.

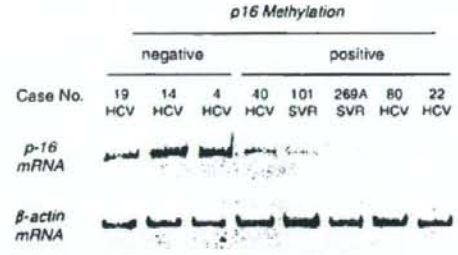

Fig. 2. Expression of *p16* mRNA in hepatocellular carcinoma (HCC). The promoter of *p16* was methylated in cases 40, 101, 269A, 80 and 22. In these tumours, *p16* expression was lower than that in HCC without methylation. β-actin expression was examined as a control. HCV, hepatitis C virus; SVR, sustained virological response.

p14, *RB* and *PTEN* are, respectively, methylated in 58–82% (4, 5, 28–32), 5–64% (5, 32–34), 0–36% (5, 32), 21% (5) and 17% (28) of HCCs from patients with viral infections. In our study, methylation of *p16*, *p15*, *p14*, *RB* and *PTEN* was, respectively, detected in 34 (77.3%), 13 (29.5%), eight (18.2%), 12 (27.3%) and 11 (25.0%) of 44 HCV-HCCs. Our findings are thus consistent with those of previous studies. In SVR-HCC, *p16* was methylated in all samples, whereas *RB* and *PTEN* were methylated in only two samples and methylation of *p15* and *p14* was not detected. This was a novel methylation profile that differed from that of SVR-HCC and HCV-HCC. We showed that promoter methylation of the *p16* gene, leading to the loss of *p16* expression, was frequently observed not only in HCV-HCC but also in SVR-HCC. These data suggested that aberrant *p16* methylation might contribute to the development of SVR-HCC.

Epidemiological studies have shown that past exposure to *Helicobacter pylori* is closely associated with an increased risk of gastric cancer and that most cases of *H. pylori*-negative gastric cancer have a history of exposure to *H. pylori* (35, 36). Maekita and colleagues reported that permanent methylation of specific CpG islands in gastric mucosae is associated with a heightened risk of gastric cancer in *H. pylori*-negative patients (37, 38). It was speculated that methylation of CpG islands in gastric stem cells led to a continuous high level of methylation in gastric mucosae (39). It was well known that HCV was spontaneously eradicated in 20% of patients with the acute infection (40). To our knowledge, there has been no report about HCC development in patients who had been cured in acute hepatitis. In the present study, *p16* was methylated in both HCC infected with HCV and HCC after eradication of HCV. We speculate that *p16* in hepatic stem cells might be methylated in the continuous presence of HCV. These cells with methylated *p16* might survive and grow after eradication of HCV by IFN therapy. Future studies should examine the methylation status of genes in successive liver specimens obtained before and after IFN therapy.

In conclusion, epigenetic alterations of some genes in SVR-HCC differed from those in HCV-HCC. Moreover, mutation of mtDNA was less common in SVR-HCC than in HCV-HCC. The present results suggest that the development of HCC in patients cured of HCV infection by IFN therapy might be associated with particular molecular alterations.

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