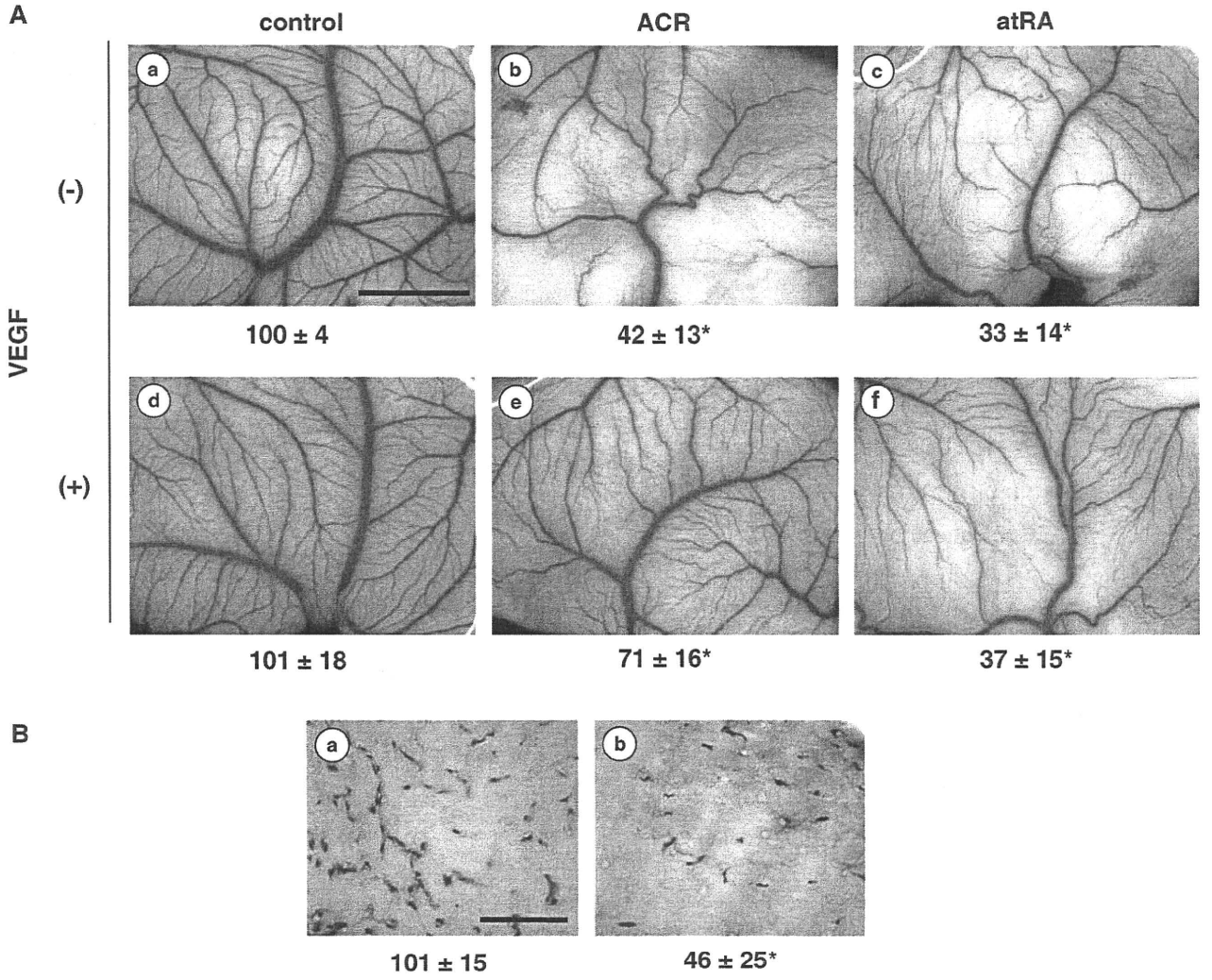

Figure 3 The antiangiogenic effect of ACR, but not of atRA, was rescued by simultaneous treatment with VEGF in CAM (**A**). The 4.5-day-old CAMs were treated with ACR and atRA for 48 h and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 3 μg/egg ACR; panel (**c**), 500 ng/egg atRA; panel (**d**), vehicle plus 1 ng/egg mouse recombinant VEGF; panel (**e**), 3 μg/egg ACR plus 1 ng/egg mouse recombinant VEGF; panel (**f**) 500 ng/egg atRA plus 1 ng/egg mouse recombinant VEGF. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 18 eggs (6 eggs per experiment × 3 experiments) were evaluated and representative results are shown. (**B**) Matrigel plug assay: matrigel plugs containing 50 ng/ml VEGF ± 5 μM ACR were implanted into mice subcutaneously. One week later, matrigel plugs were collected and stained with hematoxylin and eosin (panels a and b). Panel **a**, VEGF alone (control); panel **b**, VEGF plus ACR. Representative data from a total of nine micrographs (3 fields × 3 mice) are presented. Scale bar, 500 μm. The number of invading cells in each micrograph was counted and the relative values are presented as percentages under each photograph. An asterisk indicates a significant difference ($P < 0.05$) from the control. Panels **A** and **B** show representative results from two independent experiments, both of which gave similar results.

phosphorylation of VEGFR2 but rather increased it (compare lanes 4 with 6). Both ACR and atRA decreased the expression of VEGFR2 to about 70 and 60%, respectively, without VEGF treatment (compare lanes 1 with 2 and 3). However, this effect was not obvious in cells treated with VEGF (compare lanes 4–6). ACR did not affect the binding of VEGF to VEGFR2, nor did it affect *VEGF* mRNA levels (data not shown). On the other hand, pretreatment with ACR or atRA did not block the phosphorylation of FGFR1 but rather enhanced it (Figure 5b). Whereas ACR inhibited the phosphorylation of Ras, it did not inhibit the phosphorylation of Akt (Supplementary Figure 2). In addition, pretreatment with 5 μM ACR, but not with atRA, significantly inhibited the phosphorylation of ERK, which is induced downstream of VEGF stimuli (Figure 6a, lanes 5 and 6 in upper panel, respectively). The inhibition by ACR was inverted by the overexpression of constitutively active MAPK kinase in HUVECs (Figure 6b, lane 4 in upper panel).

Effect of ACR and atRA on HCC-Induced Angiogenesis in a Xenografted CAM Model

To confirm whether ACR and atRA have anti-HCC-induced angiogenic activity *in vivo*, we investigated the effect of ACR and atRA on HCC-induced angiogenesis in a xenografted

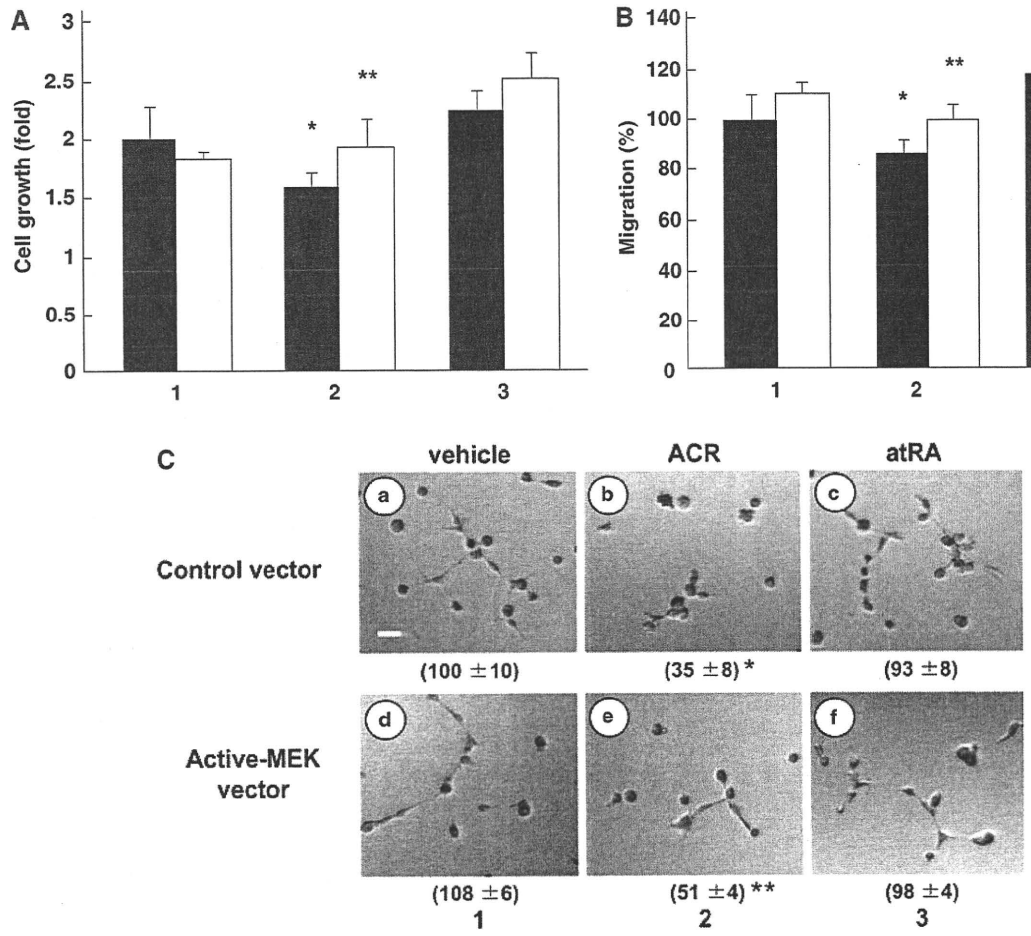

Figure 4 Effects of ACR and atRA on endothelial cell growth, migration, and tube formation. (**A**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells (1×10^5 cells) were seeded onto 3.5-cm dishes and incubated for another 2 days. They were then incubated for 48 h in α-MEM medium containing 10% fetal calf serum, 100 ng/ml VEGF, and 5 μM ACR or atRA. Cells were counted and cell numbers are plotted as ploidy relative to values for untreated control cells at the start of incubation with ACR or atRA. Values represent means ± s.e. ($n=2$). Lane 1, vehicle (0.1% ethanol); lane 2, ACR; lane 3, atRA. Closed columns, cells overexpressing control vector; open columns, cells overexpressing constitutively active MAPK kinase. A single asterisk indicates a significant difference ($P<0.05$) from the control (lane 1, closed column) and double asterisks indicate a significant difference ($P<0.05$) between samples with or without the overexpression of constitutively active MAPK kinase. (**B**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells were wounded with a tip of pipette and incubated for 12 h in α-MEM medium containing 2.5% fetal calf serum, 100 ng/ml VEGF, and 5 μM ACR or atRA. The numbers of cells that migrated into the denuded area were counted and are plotted as percentages relative to values for untreated control cells. Values represent means ± s.e. ($n=2$). Lane 1, vehicle (0.1% ethanol); lane 2, ACR; lane 3, atRA. Closed columns, cells overexpressing control vector; open columns, cells overexpressing constitutively active MAPK kinase. A single asterisk indicates a significant difference ($P<0.05$) from the control (lane 1, closed column) and double asterisks indicate a significant difference ($P<0.05$) between samples without or with the overexpression of constitutively active MAPK kinase. (**C**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells were seeded onto polymerized matrigel at 2×10^5 cells/well. Thereafter, 100 ng/ml VEGF and 5 μM ACR or atRA were added, and incubated for 6 h. Patterns of tube formation were photographed. Scale bar, 100 μm. Panels (**a**–**c**), cells overexpressing control vector; panels (**d**–**f**), cells overexpressing constitutively active MAPK kinase. Panels (**a** and **d**), vehicle (0.1% ethanol) plus 100 ng/ml VEGF; panels (**b** and **e**), 5 μM ACR plus 100 ng/ml VEGF; panels (**c** and **f**), 5 μM atRA plus 100 ng/ml VEGF. The numbers of branches in each micrograph were counted and the relative values are presented as percentages under each photograph. A single asterisk indicates a significant difference ($P<0.05$) from the control (panel **a** *vs* panel **b**) and double asterisks indicate a significant difference ($P<0.05$) between samples with or without the overexpression of constitutively active MAPK kinase (panel **b** *vs* panel **e**). Panels **A**–**C** show representative results from two independent experiments, both of which gave similar results.

CAM model (Figure 7). Although 5 μM ACR inhibited HCC-induced angiogenesis by 37% (panel b), the same concentration of atRA did not show any inhibition at all (panel c). This result suggested that ACR, but not atRA, may prevent the recurrence of HCC in part through the inhibition of cancer angiogenesis.

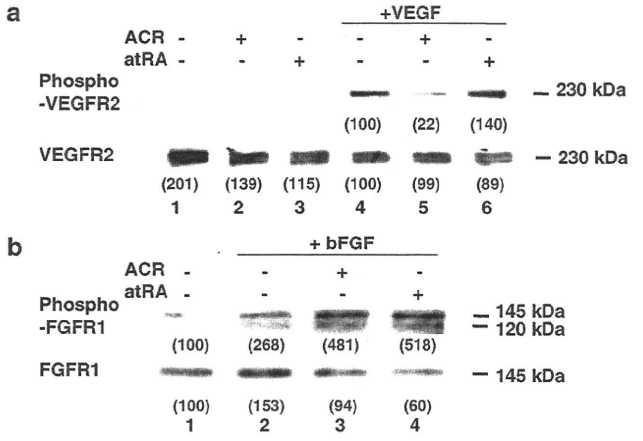

Figure 5 Effects of ACR and atRA on phosphorylation of growth factor receptors. After HUVECs had been incubated for 24 h with or without 5 μM ACR or atRA in medium containing 2.5% serum, cells were stimulated with either 100 ng/ml VEGF (panel **a**) or 50 ng/ml bFGF (panel **b**) for 5 min, and then lysed immediately. The amounts of each phosphorylated receptor (each upper bands), as well as whole amounts of each receptor (each lower bands), were assessed as described in the Materials and methods section. Panels **a** and **b** show representative results from two independent experiments, both of which gave similar results.

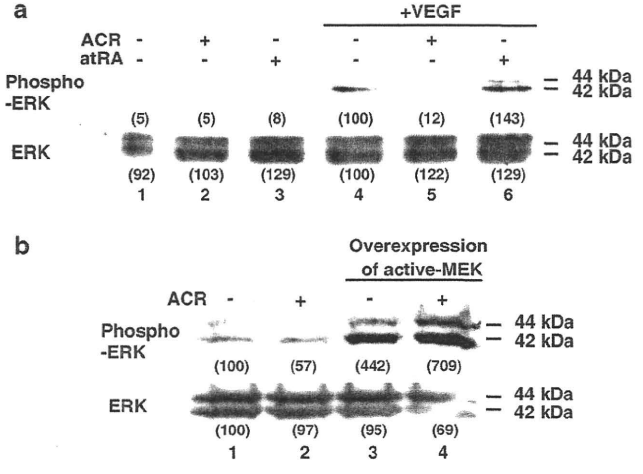

Figure 6 Effect of ACR on phosphorylation of ERK. (**a**) After HUVECs had been incubated for 24 h with or without 5 μM ACR or atRA in medium containing 2.5% serum, cells were stimulated with 100 ng/ml VEGF for 5 min and then lysed immediately. (**b**) HUVECs were transfected with a constitutively active *MEK* gene. The day after transfection, the medium was changed and cells were treated with 5 μM ACR and lysed immediately. The amounts of each phosphorylated ERK (upper bands), as well as whole amounts of ERK (lower bands), were assessed as described in the Materials and methods section.

DISCUSSION

In this paper, we have shown an antiangiogenic activity of ACR and its underlying molecular mechanism, differing from that of atRA. Although the relative antiangiogenic activity of ACR was about 10 times weaker than that of atRA at the same concentrations (Figure 1), ACR showed much stronger inhibition in endothelial cell growth, migration, and tube formation than atRA (Figure 4) because of suppression in the VEGF-MAPK pathway (Figures 5 and 6). ACR suppressed the phosphorylation of VEGFR2 (Figure 5a, lane 5). On the other hand, atRA induced the phosphorylation of VEGFR2 (Figure 5a, lane 6). This might be because of the induction of VEGF by atRA as reported previously.[17] ACR did not affect the levels of *VEGF* mRNA and the transactivation of the *VEGF* promoter (data not shown). ACR slightly inhibited the phosphorylation of VEGFR1 at 300 μM but not at all at 30 μM (Ishibashi *et al*, unpublished data). ACR (1 or 10 μM) did not inhibit other tyrosine kinases (for example, EGFR, FGFR3, FLT3, IGF1R, MET, PDGFR-α, PDGFR-β, and TRKB) (Ishibashi *et al*, unpublished data). Moreover, ACR inhibited the phosphorylation of Ras but not the phosphorylation of Akt (Supplementary Figure 2). Therefore, we speculate that ACR may selectively interfere with the phosphorylation of VEGFR2 after Ras activation, although it is not clear how ACR does this; the underlying detailed molecular mechanism(s) remain to be elucidated. ACR and atRA enhanced the phosphorylation of FGFR1 (Figure 5b, lanes 3 and 4). This result might be because of the induction of bFGF by atRA as previously reported.[18] ACR might also induce bFGF, probably through its retinoid activity.

These results suggest that suppression of the recurrence of HCC by ACR was induced in part through its antiangiogenic property by directly suppressing endothelial growth, migration, and tube formation through inhibition of the VEGFR2-MAPK axis. On the other hand, atRA inhibits angiogenesis by a disruption of vascular networks through an increased expression of Ang2 and inhibition in the Ang/Tie2 pathway.[6] Compared with atRA, ACR has 1/10–1/100 weaker 'retinoid' activities.[19] It has a 1/10 weaker action on leukemia differentiation,[20] and a 1/100 weaker carcinogenic action in transgenic mice that express the dominant-negative form of retinoic acid receptor.[21,22] On the other hand, accumulating evidence shows that ACR, but not atRA, has apoptosis-inducing activity in HCC cells, as well as in smooth muscle cells and vascular neointima.[23,24] We found that ACR acts as either a kinase inhibitor or a phosphatase stimulator and prevents hyperphosphorylation of RXR,[9] and now VEGFR2. In this context, we speculate that ACR resembles geranylgeraniol in terms of its isoprenoid-like structure, which has been implicated in the modulation of many phosphorylation/dephosphorylation signaling.[25,26]

Hepatocellular carcinoma is a major cause of cancer mortality worldwide, especially in Southeast Asia and in sub-Saharan Africa.[27] The development of HCC is frequently associated with a chronic inflammation of the liver induced by persistent infection with hepatitis B virus or hepatitis C virus. The annual incidence rises to ~20–25% in cirrhotic patients who have undergone a potentially curative removal of primary HCC in Japan; the recurrence rate at 5 years after the curative treatment may exceed 70%. This high recurrence rate is not because of local recurrence or metastasis from the original lesion, but rather from a second primary lesion.[19]

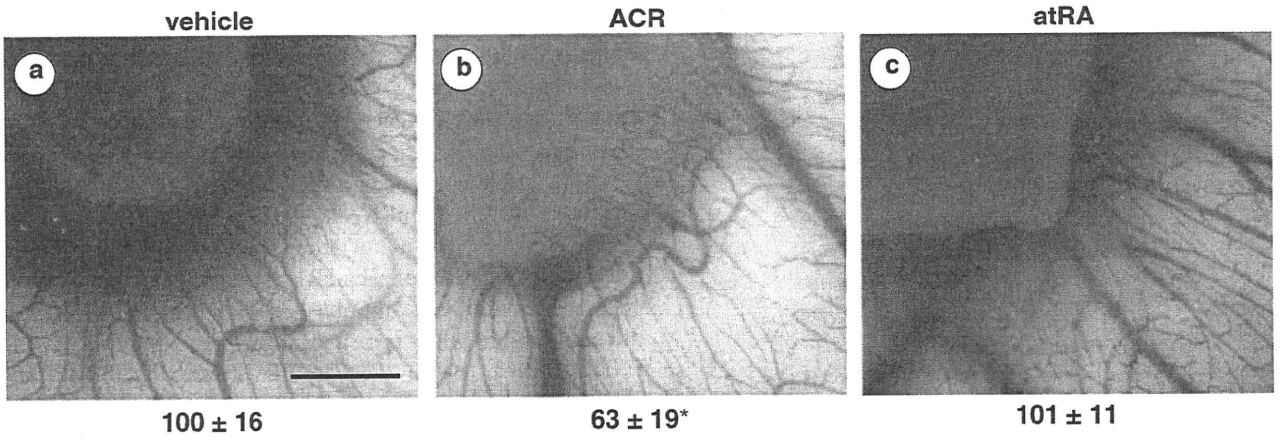

Figure 7 Effect of ACR on HCC-induced angiogenesis on CAM. HepG2 cell suspensions with or without 5 μM ACR or atRA were delivered at 4×10^5 cells per embryo onto the top of CAM on day 8 using a gelatin sponge implant. After a further 4-day incubation, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid, and patterns of angiogenesis toward the implant were photographed. Panel (**a**), vehicle (1% EtOH); panel (**b**), 5 μM ACR; panel (**c**), 5 μM atRA. Scale bar, 1 mm. Total numbers of branches of blood vessels were counted and the relative values are presented as percentages under each photograph. An asterisk indicates a significant difference ($P<0.05$) from the control. This result shows the representative result from two independent experiments, both of which gave similar results.

In HCC tissues, RXR-α is constitutively phosphorylated by the activation of MAPK, resulting in a loss of its function and accumulation of inactive RXR-αs inside cells as dominant-negative inhibitors.[9,10] Therefore, phosphorylation of RXR-α causes a reduction in transactivation through a RAR/RXR complex.[9,28] ACR inhibits the phosphorylation of RXR-α and restores the function of RXR-α, and thereby transactivation through the RAR/RXR complex with endogenous retinoic acid. Retinoids are thought to activate the transcription of cell cycle inhibitor $p21^{CIP1}$ by RAR[29] and by apoptosis-inducer tissue transglutaminase in HCC.[19] However, 5 μM ACR hardly induces endothelial cell death (Figure 4A) and expression of $p21^{CIP1}$ mRNA levels in endothelial cells (data not shown). We found that phosphorylation of RAR/RXR was associated with the growth of endothelial cells and that ACR inhibited this phosphorylation (Supplementary Figure 3). As this phosphorylation of RAR/RXR coincided with the activation of VEGFR2 and Ras and as we had already found that RAR/RXR phosphorylation was induced by the overexpression of active MEK in cancer cells,[10] we speculate that phosphorylation of RAR/RXR would occur downstream of the VEGF/VEGFR axis in growing endothelial cells.

Acyclic retinoid suppressed both angiogenesis and HCC-induced angiogenesis in a xenografted CAM model (Figure 1, panels b–d and Figure 7, panel b, respectively). In this model, ACR did not affect the preexisting blood vessels on CAM. These results suggest that ACR did not affect the mature blood vessel and only affected neovasculature formation, including both normal angiogenesis and tumor angiogenesis. On the other hand, atRA suppressed angiogenesis but failed to suppress the tumor angiogenesis on CAM (Figures 1 and 7, panels e–g and panel c, respectively), suggesting that atRA inhibits angiogenesis in the embryonic stage, but not tumor-induced angiogenesis, and addressing the superiority of ACR as a promising antitumor angiogenesis agent. The dose of ACR used in this experiment (5 μM) is higher than the maximal blood concentration of ACR in clinical use (1 μM) (Ishibashi *et al*, unpublished data). However, we believe that liver tissue has higher doses of ACR than does the blood level because of the accumulation of ACR in the liver. These results suggest that one of the mechanisms of ACR action to prevent recurrence of HCC is its antiangiogenic activity.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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分冊2

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肝炎等克服緊急対策研究事業（肝炎分野）

ウイルス性肝炎における最新の治療法の標準化を目指す研究に関する研究

平成22年度　総括・分担研究報告書

研究代表者　　熊田　博光

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Review Article

Predictors of Virological Response to a Combination Therapy with Pegylated Interferon Plus Ribavirin Including Virus and Host Factors

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A combination therapy with pegylated interferon (PEG-IFN) plus ribavirin (RBV) has made it possible to achieve a sustained virological response (SVR) of 50% in refractory cases with genotype 1b and high levels of plasma HCVRNA. Several factors including virus mutation and host factors such as age, gender, fibrosis of the liver, lipid metabolism, innate immunity, and single nucleotide polymorphism (SNPs) are reported to be correlated to therapeutic effects. However, it is difficult to determine which factor is the most important predictor for an individual patient. Data mining analysis is useful for combining all these together to predict the therapeutic effects. It is important to analyze blood tests and to predict therapeutic effects prior to initiating treatment. Since new anti-HCV agents are under development, it will be necessary in the future to select the patients who have a high possibility of achieving SVR if treatment is performed with standard regimen.

1. Progress in Virological Response in the Difficult-to-Treat Patients with Genotype 1 Hepatitis C Virus (HCV) Infection and Factors Correlated to the Efficacy

Recently, the average age of the patients with chronic hepatitis C has been increasing in Japan. Incidence of hepatocellular carcinoma (HCC) in the elderly patients with chronic hepatitis C (65 years or older) has demonstrated to be higher than younger ones when adjusted by the stage of hepatic fibrosis [1]. In Japan, refractory cases with genotype 1b and high HCVRNA levels are seen in as high as 70 percent of chronic hepatitis C patients. The outcome of conventional IFN monotherapy has been an SVR response of 3%–5% after 6 months of treatment in genotype 1b and high HCVRNA patients [2, 3], and virus mutation such as interferon sensitivity-determining region (ISDR) is shown to be correlated with the virological response [2]. The association of ISDR mutations and virological response to IFN monotherapy was denied in an Italian study [4]; however, it was confirmed by a Chinese study [5] and an international meta-analysis [6].

However, pegylated IFN (PEG-IFN) extends the duration of therapy and reduces adverse effects, and for this reason, PEG-IFN has become the cornerstone of therapy. Furthermore, by the combination therapy with PEG-IFN and ribavirin (RBV), the rate of SVR has dramatically improved. Even in the patients with genotype 1b and high HCVRNA level, SVR rate reaches as high as 40%–50%, thereby improving the therapeutic effects both in Western countries [7, 8] and in Japan [9, 10].

It is important to predict the rate of achieving SVR in the individual patient, before initiating treatment. Both virus- and host-related elements have been reported as factors correlated to therapeutic effects of combination therapy [11–13]. A particular focus has been placed on virus mutations, age, gender, fibrosis of the liver, lipid metabolism, and degree of fatty metamorphosis of the liver.

Among these factors related to PEG-IFN and RBV, innate immunity has been shown to be correlated in virological

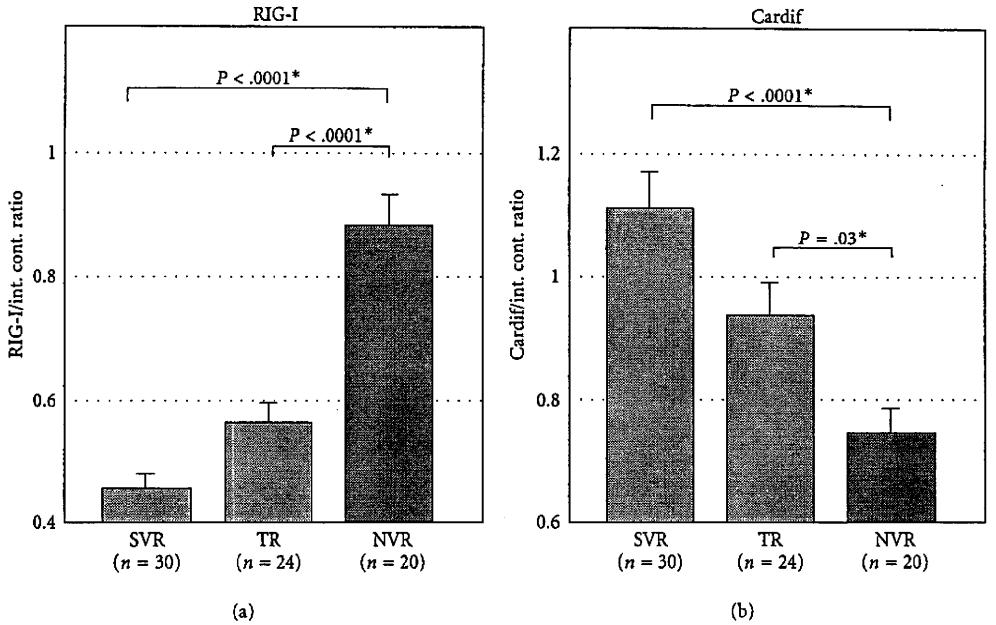

FIGURE 1: Expression of genes correlated to the intrahepatic innate immunity and virological response to PEG-IFN alpha-2b and RBV combination therapy. Open column indicates SVR (n = 30), gray column indicates TR (n = 24), and closed column indicates NVR (n = 20). Error bars indicate the standard error. The P values were analyzed by the Kruskal-Wallis test. Expression of Rig-I was significantly higher in NVR than in SVR patients, and Cardif expression was higher in SVR than in NVR. The figure was cited from [8].

response. Asahina et al. reported that liver biopsies were performed before the PEG-IFN and RBV combination therapy to examine the correlation between the gene expression involved in innate immunity and the therapeutic effects, and in the patients in whom RIG-I expression is high and the expression of Cardif, an adaptor gene, is low, it was found that there are many nonresponders (NVRs) in which HCVRNA does not become negative during the course of treatment [13]. It was therefore revealed that there are many NVRs among the patients in whom the ratio of RIG-I to Cardif in liver tissue is high and that this ratio is low in the SVR patients. Based on these findings, it has become clear that innate immunity is correlated to therapeutic effects (Figure 1).

Furthermore, it was recently discovered that a single nucleotide polymorphism (SNP) of the host gene IL28B is significantly involved in the therapeutic response to the PEG-IFN and RBV combination therapy [14, 15]. The possibility of becoming an NVR is high in cases of the minor allele carriers of IL28B. However, it is not possible to routinely measure an SNP of IL28B in the clinical setting. In this paper, factors which can actually be used in real clinical practice are discussed for the prediction of the efficacy of PEG-IFN and RBV combination therapy.

2. Amount of HCVRNA

In the patients with chronic hepatitis C, it is not possible to directly measure the amount of virus, and the amount of HCVRNA is measured instead. Currently, a real-time PCR method which has an advantage of wide range and high sensitivity is utilized, and measurements can be taken from a single blood sample of a very small amount, that is, 1.2 log copies/ml, to a very large amount, that is, 8 log copies/ml. This method has a higher level of sensitivity than the conventional Amplicor monitor test and can therefore detect HCVRNA even if only a very small amount exists in the plasma. If the amount of HCVRNA in plasma is less than the range of sensitivity of the real-time PCR method, it is recorded as undetectable level. If the indication is "less than 1.2 log copies/ml of HCVRNA", it means that a very small amount of HCVRNA exists in the plasma. Since the indication of the real-time PCR method is based on log counts, a decrease of 1.0 in the numerical value means that the amount of HCVRNA has decreased to 1/10. With the application of this real-time PCR method, it has become possible to measure amounts of HCVRNA up to 8 log copies/ml, and it has also become possible to predict the efficacy before treatment and to monitor appropriately the reactivity during the course of treatment. However, in the patients in whom a PEG-IFN and RBV combination therapy is performed, SVR can be acquired even when the amount of virus prior to the treatment is quite large. It is therefore difficult to predict the virological response solely from the amount of HCVRNA before starting the treatment. Once treatment has commenced, at what week HCVRNA becomes negative is important for the prediction of therapeutic effects, and this serves as a parameter for deciding the duration of treatment [16].

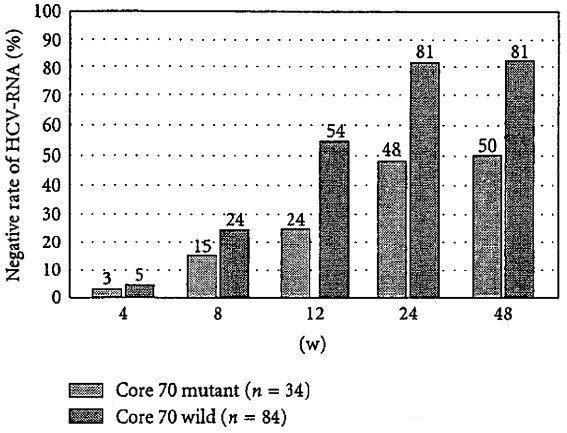

FIGURE 2: Comparison of aa70 mutations in the HCV core region and the rate of HCVRNA becoming negative during the course of treatment. Compared with the wild type, among the patients of aa70 mutations, there were fewer patients in whom HCVRNA had become negative during the course of treatment.

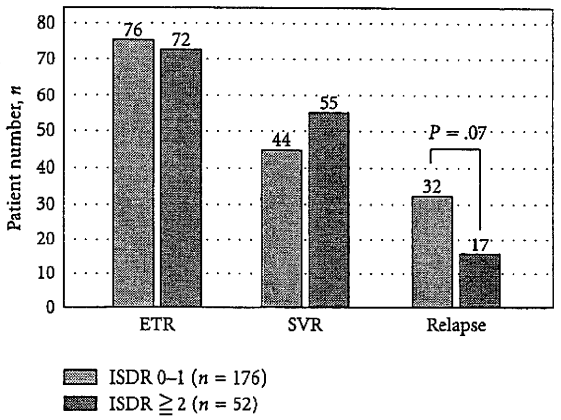

FIGURE 3: Number of ISDR substitutions and the comparison of virological response, SVR, and relapse at the end of the treatment. Compared with the patients with 2 or more sites of substitutions, the rate of SVR was lower and the rate of relapse was higher in the patients in whom there were fewer substitutions in ISDR, that is, 0 or 1 sites.

Measuring the rate of viral clearance from serum is helpful for predicting the likelihood of a response to PGEIFN and RBV and useful for determining the optimal duration of therapy if the patients start to receive the treatment [17]. In the AASLD practice guideline, response-guided therapy is highly recommended [18]. In two nationwide registration trials conducted in Japan [9, 10], the SVR rate was high, from 76% to 100%, in patients whose HCVRNA was cleared rapidly from serum by week 4 (rapid virological response; RVR), and 71% to 73% in patients who achieved undetectable HCVRNA from week 5 to week 12 (early virological response; EVR). In contrast, the SVR rate in patients with late clearance of HCVRNA from week 13 to week 24 was low at 29% to 36%. No patients without clearance of HCVRNA by week 24 achieved SVR.

The strategy of extending therapy in patients with delayed virological responses, defined as clearance of HCV-RNA between weeks 12 and 24, was evaluated in five studies [19–23]. These results cannot be compared directly with each other because of the heterogeneous study populations, differences in the baseline characteristics, and the different regimens utilized amongst them. Nevertheless, the results showed a trend toward a higher SVR rate by extending therapy from 48 to 72 weeks in patients with delayed virological response.

3. Viral Mutations in Core and NS5A Region

In the patients with genotype 1b HCV infection, the mutations in aa70 and aa91 in the core region have been shown to correlate with early virological response (EVR) during PEG-IFN and RBV combination treatment [11]. If aa70 in the core region is mutated to anything other than arginine and aa91 to anything other than leucine, it is difficult to achieve EVR, and it is thus highly possible that such cases will become nonresponders. The examination results at our institution including 292 patients with genotype 1b infection demonstrated that, in the cases with mutations in aa70 in the core region, the rate of HCVRNA becoming negative during the course of combination treatment was low compared to the wild type of aa70 (Figure 2). However, core aa70 mutation is shown to have quasispecies detectable by cloning, and 70Q clone was positively selected during combination treatment with PEGIFN and RBV [24].

Furthermore, Enomoto et al. reported that the patients with 4 or more amino acid mutations were observed in interferon sensitivity-determining region (ISDR) within NS5A region [2]; SVR rate is higher than 90% by IFN monotherapy, and SVR is less than 10% in the patients with no mutation in ISDR. It has also been reported that, in PEG-IFN and RBV combination therapy, the number of ISDR mutations is involved in the SVR [12].

We analyzed the relationship between virological response and ISDR mutations in the patients with genotype 1b infection treated by PEG-IFN alpha-2b and BRV combination therapy. In the patients with 0 or 1 ISDR mutation, even if the rate of HCVRNA becoming negative at the end of treatment was the same, the rate of SVR would be lower compared with the patients having 2 or more mutations (Figure 3). This demonstrates that there is a higher incidence of relapse after the end of treatment in the patients with 0 or 1 ISDR mutation.

Enomoto and Maekawa reported that mutations both in NS5A-ISDR (interferon sensitivity-determining region) and core 70Q substitution are associated with no early viral response during PEGIFN and RBV combination therapy [25]. Association of core aa70 substitution and mutations in NS5A region is confirmed to be associated with viral response by PEGIFN and RBV combination therapy in a Japanese multicenter cooperative study [26]. The number of

mutations in the interferon sensitivity-determining region was shown to be associated with the viral response to PEGIFN and RBV combination treatment not only in Japan [27], but also in Tunisia [28].

Recently, a consensus has been established that mutations in aa70 in the core region are important for the prediction of HCVRNA becoming negative during the early course of treatment, and the number of ISDR mutations is important for the prediction of relapse after the end of treatment.

4. Adherence

It has been confirmed that it is important to ensure 80% or more of the scheduled dose of both PEG-IFN and BBV in order to improve the rate of SVR, and together with the duration of treatment, the 80 · 80 · 80 rule has been established. However, Schiffman et al. recently reported that the dose of PEG-IFN in the initial stage of administration is important and that, if a sufficient dose of PEG-IFN is administered, then 60% or more of the RVB dose would be enough [29]. It is therefore of primary importance to ensure the dose of PEG-IFN.

In Japan, the average age of patients with chronic hepatitis C is increasing, and achieving good adherence is difficult in many patients. Consequently, the rate of SVR is low in elderly patients. How to improve the rate of SVR in elderly patients is an important issue. With regard to the dose of RBV, reducing the RBV dose based on the calculation of the total body clearance (CL/F) has been proposed to be useful for decreasing the discontinuation and improving the rate of SVR. Although there is no consensus on an appropriate dose of PEG-IFN in elderly patients, if the initial dose is set lower than the usual dose, discontinuation would be reduced. Thus, it is necessary to investigate whether such an initial dose would improve the rate of SVR.

Recently, the risk of hemolytic anemia was clearly demonstrated to correlate with ITAP gene SNP [30]. The predictive implication should be analyzed prospectively in clinical practice.

5. Host Factors

Zeuzem et al. described the factors related to the less response to interferon-based therapy, and he showed that several host factors such as older age, race, and obesity are responsible factors for the poor response to IFN [31]. Recently, insulin resistance which was examined by homeostasis model assessment index (HOMA-IR) was shown to be associated with a lower rate of SVR, and body mass index (BMI) was not identified as a significant factor for the poor response to PEGIFN and ribavirin combination therapy [32]. Insulin resistance was confirmed as a related factor to the nonresponse to interferon-based treatment [33]. However, Charlton et al. reported that obesity itself is an associated factor for decreased efficacy of interferon-based therapies, and they discussed the possible mechanism [34], and obesity was shown to be associated with the increased enhancement of suppressor of cytokine signaling (SOCS) family in the hepatocytes [35].

6. Data Mining Analysis

Both virus- and host-related factors are correlated to therapeutic effects of PEG-IFN and RBV. One important question is which of these factors should be focused on in order to predict the therapeutic effects in an individual patient. In addition, in each individual patient, the host and virological factors are different. It is therefore difficult to predict the virological response in each case before treatment. Furthermore, although it is important to predict the relapse rate when HCVRNA becomes within an undetectable level in an individual patient, prediction of the rate of SVR including virological and host factors and adherence to the treatment has never been carried out in an individual patient.

A data mining analysis is the process of analyzing a large amount of data by a computer in order to develop an algorithm. Conventional statics have been used to examine certain hypothesis. Data mining is superior in that it can set an algorithm, using a computer, based on a large amount of data without a hypothesis.

We therefore conducted at our institute a data mining analysis of the patients with genotype 1b infection having high levels of HCVRNA to whom a PEG-IFN alpha-2b and RBV combination therapy was administered to investigate whether by the 12th week after the commencement of treatment HCVRNA became negative (EVR) (Figure 4) [36]. The most important factor for the prediction of EVR was the steatosis of the liver: when steatosis was observed in 30% or more of hepatocytes, EVR was found to be difficult to achieve. In the patients in whom steatosis was not severe, the second most important factor was the serum LDL cholesterol value. While the rate of EVR was 57% in the patients in whom this value was 100 mg/ml or above, the rate of EVR was 32% in the patients in whom the LDL cholesterol was less than 100 mg/dl.

The higher the LDL cholesterol value, the earlier the HCVRNA became negative. Among the patients with low LDL cholesterol values, while the rate of EVR was 15% in patients 60 years of age or older, the rate was high in the patients under the age of 60 years old, that is, 49%. Among patients under the age of 60, the rate of EVR was low, that is, 31%, in patients with a blood glucose level of 120 mg/dl or above whereas EVR was achieved in 71% of the patients with a blood glucose level of less than 120 mg/dl (Figure 4).

On the other hand, in the patients with high LDL cholesterol values, the next most important factor was age. While the rate of EVR was 50% in patients 50 years of age or older, EVR was achieved in 77% of the patients under the age of 50. Among patients of 50 years of age or older, the next most important factor was the gamma GTP value. While the rate of EVR was 35% in the patients in whom gamma GTP was 40 IU/L or above, EVR was achieved in 60% of the patients where the value was less than 40 IU/L.

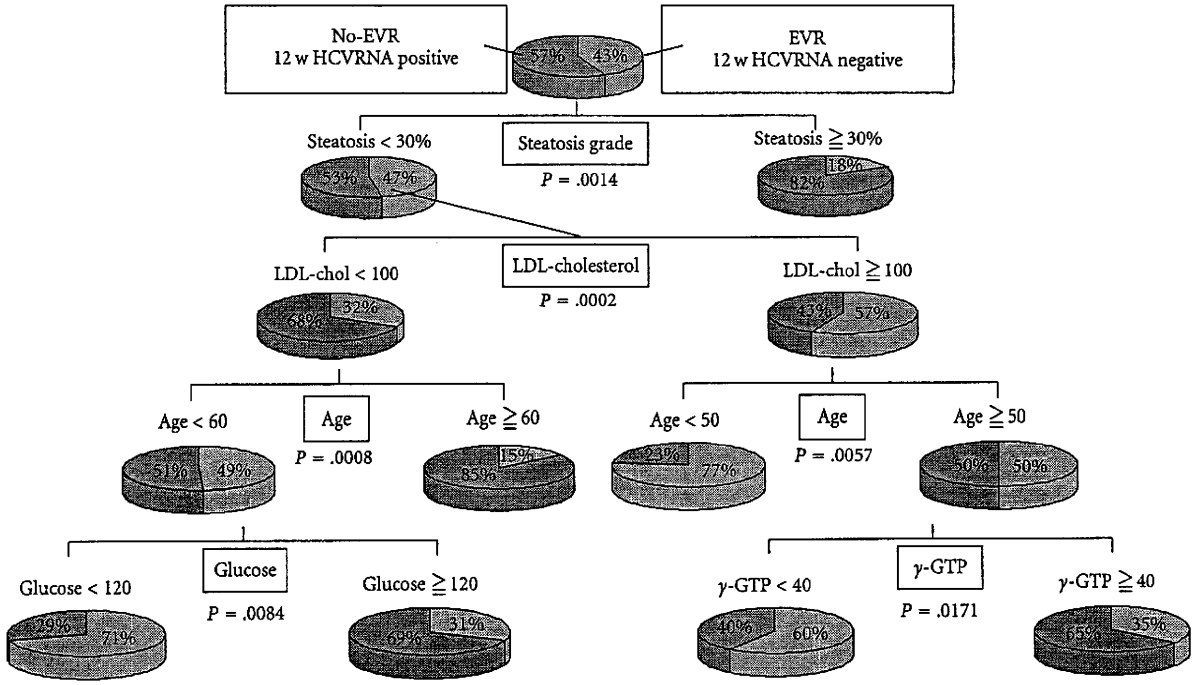

FIGURE 4: HCVRNA negative (EVR) algorithm at 12th week from data mining analysis of PEG-IFN alpha-2b plus RBV combination in the genotype 1b and high levels of HCVRNA. Both virological and host factors were evaluated by data mining analysis software from SPSS. This figure was cited from [36].

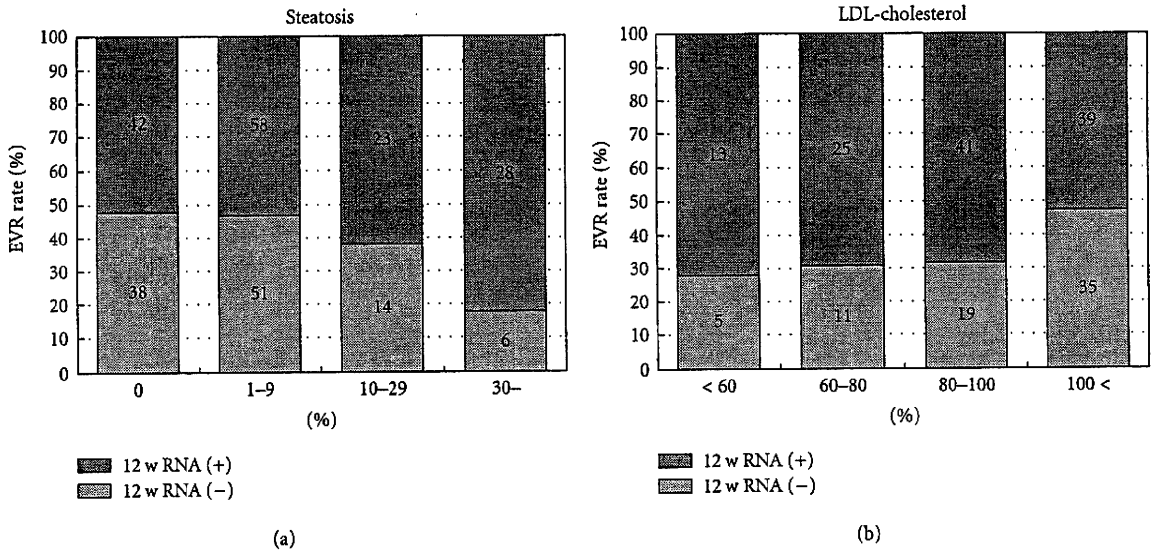

FIGURE 5: The rate of EVR in the patients with genotype 1b and high levels of HCVRNA, based on fatty deposition in the liver (a), and the LDL cholesterol value (b), respectively. EVR was highly achieved in the patients with less steatosis in the liver, and in those with high serum LDL-cholesterol levels. This is univariate analysis, and cited from [36].

We therefore compared these factors based on the original data. A univariate comparison of the fatty infiltration of the liver and the rate of EVR demonstrated that the rate of EVR was higher when the steatosis of the liver was less severe (Figure 5(a)). In addition, a comparison of the LDL cholesterol value and the rate of EVR demonstrated a significant correlation, confirming that the higher the LDL cholesterol value, the higher the rate of EVR (Figure 5(b)). Therefore, it was also proposed by the results of univariate analysis of each factor extracted from the data mining analysis that these factors were correlated to the rate of EVR.

From these observations, it is likely to improve the viral response to PEGIFN and ribavirin by reducing steatosis of the liver through daily walking or abstinating alcohol intake or by refraining from high-fat diet.

By utilizing data mining, it is therefore possible to assess virus- and host-related factors together and to predict the virological response in each patient, and thereby clinically useful information can be obtained. The algorithm should be validated including a large number of the patients.

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REVIEW

Recent advances of radiofrequency ablation for early hepatocellular carcinoma

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Key words
Hepatocellular carcinoma, radiofrequency ablation, seeding, transarterial chemoembolization, CLIP, BCLC, JIS, des-gamma-crboxy prothrombin time (DCP), alpha-fetoprotein (AFP).

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Conflict of interest
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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of death in the malignant neoplastic diseases in the world. Surgical operation is sometimes not indicated because of complicated liver cirrhosis and extrahepatic disorders. Radiofrequency ablation has been developed as a less invasive treatment for HCC since 1999, and long-term outcome has been shown. There are several complications which should be paid attention, and to improve the prognosis, combination treatment with transarterial chemoembolization should be discussed. Overall survival after between RFA and surgical resection should be compared prospectively. Establishment of staging system for treatment allocation of HCC and prevention of HCC recurrence is important issue to be examined.

Introduction

Radiofrequency ablation (RFA) has been utilized as a less invasive and curative treatment for the treatment of hepatocellular carcinoma (HCC), and the methods and procedure have been developed. In some countries, it has been chosen as first line treatment for early stage HCC. Long-term prognosis has been reported and the associated factors for the prognosis after RFA have been shown. Several complications were reported after RFA. The prognosis was compared between patients who were treated by between surgical resection and those treated by RFA. The recent developments and future perspective of RFA is discussed in this review.

Radiofrequency ablation method

Of all therapeutic apparatus compared and evaluated up to now, the RF 3000 generator system (Boston Scientific, Boston, USA) had the most positive therapeutic effects.[1] However, in many articles, an internally cooled single electrode was used.[2] When there was a risk of RFA incurred by the hepatocellular carcinoma (HCC) location, the therapeutic effects were reduced, in particular the complete response rate was low in the vicinity of the gall bladder and the stomach and intestine, as well as the diaphragm, and in the vicinity of large blood vessels.[3] However, it has been reported that, although the therapeutic effects are not reduced when tumors exist in the vicinity of large blood vessels or adjacent to the extrahepatic organs, attention should be paid to the prevention and control of complications.[4] RFA with the use of artificial ascites for HCC adjacent to the diaphragm and to the stomach and intestine produced sufficient therapeutic effects, thereby improving the sonic window.[5]

When performing RFA, the use of a guiding needle with an external insulated sheath was useful because it allowed for precise tumor targeting.[6] The use of laparoscopic RFA has allowed a sufficient therapeutic effectiveness to achieve complete tumor ablation in all cases when the HCC nodule is located with bulging or at subcapsular area, as well as an adequate safety margin, compared to percutaneous RFA.[7] As shown in Fig. 1, extra-hepatic protruding HCC nodule is the most appropriate indication for laparoscopic RFA, and complete necrosis could be achieved after one treatment session under laparoscopic ultrasound guiding. When RFA was performed under laparoscopy, complete necrosis is usually observed.[8]

Assessment of the therapeutic effect of RFA

Although the effect of RFA is, in general, evaluated by dynamic computed tomography (CT) scans taken 1 to 7 days after the procedure, it was possible to assess the therapeutic effect by multidetector row helical CT (MD-CT) immediately after RFA,

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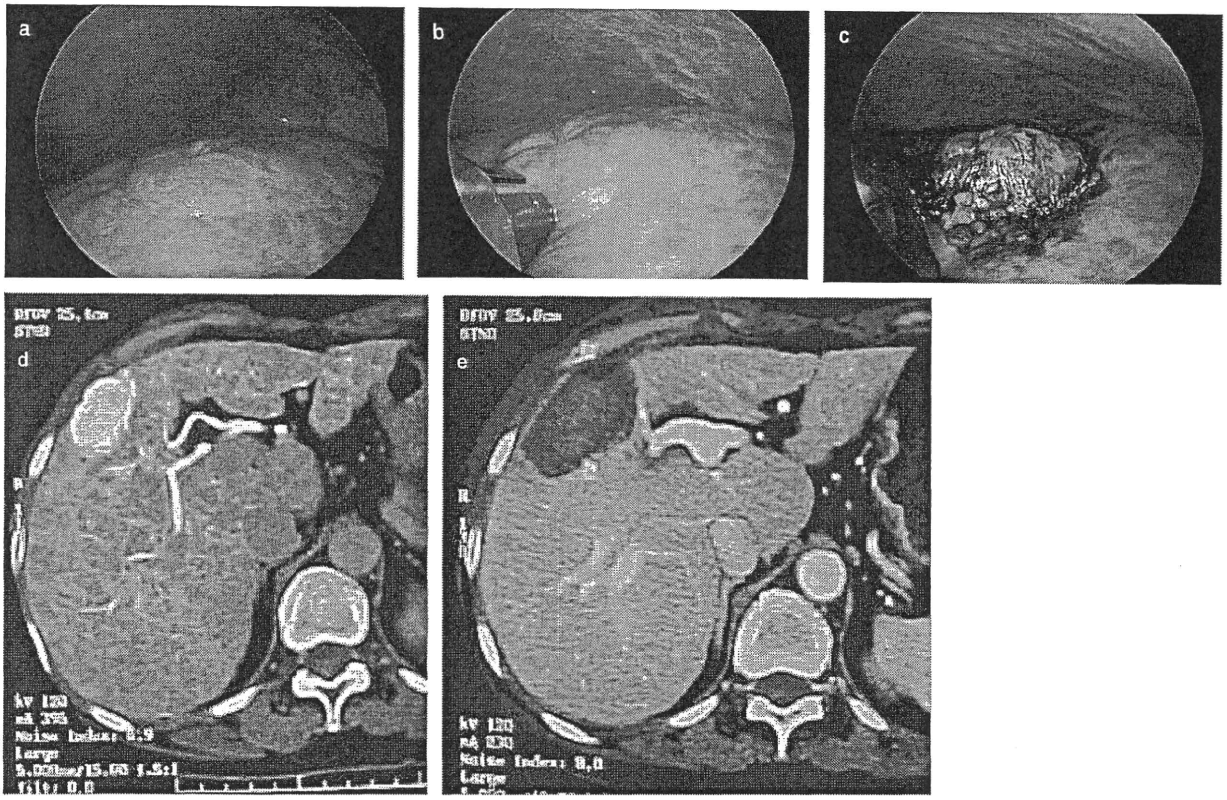

Figure 1 Hepatocellular carcinoma (HCC) nodule protruding from the liver surface is treated by laparoscopic radiofrequency ablation (RFA) under ultrasound guiding. (a) HCC nodule is directly observed under laparoscopy. (b) Under laparoscopic ultrasound guiding, RFA electrode is introduced to accurate position of the nodule, avoiding damage to diaphragm and intrahepatic vessels. (c) The entire HCC nodule was completely ablated by RFA. (d) Computed tomography (CT) scan before the treatment revealed hypervascular nodule with 2.6 cm in diameter at the surface of the liver. (e) After laparoscopic RFA, complete necrosis was confirmed by CT scan.

thereby achieving shorter hospital stays.[9] As well, one report indicates that it was possible to assess the therapeutic effect by contrast-enhanced ultrasonography immediately after RFA.[10] Contrast-enhanced sonography with abdominal virtual sonography was useful in monitoring the therapeutic effect and reducing the CT scan frequency.[11]

Prognosis after radiofrequency ablation

According to a report from a single institution in France, RFA was performed in 235 cases, with up to three HCC ≤ 5 cm in diameter, and achieved complete ablation in 222 cases. 67 cases were judged potentially resectable according to Barcelona Clinical Liver Cancer (BCLC) criteria; in these patients, RFA treatment produced 76% survival at 5 years. The factors contributing to survival were the prothrombin time and serum alpha-feto protein (AFP) levels. Conversely, the factors related to recurrence were multinodular tumors as well as the AFP level. In this report, RFA could be used as an effective first-line treatment in patients with a single nodule of 5 cm or less, a low serum AFP level, and well preserved liver function.[12]

According to a report from Italy, RFA was performed in 218 cases of single nodule HCC, measuring 2 cm or less in diameter, followed by an analysis of the prognosis. The 5-year survival rate was 68.5%, with a low 1.8% incidence of complications. Compared with resection, it was less invasive and could be conducted at a lower cost. It could therefore be considered the treatment of choice for resectable single HCC ≤ 2.0 cm.[13] In Japan, the prognosis of 1000 patients who had undergone RFA was analyzed; the 1, 3, and 5-year survival rates were 94.7%, 77.7% and 54.3%, respectively.[2] According to a report from China, the factors related to the prognosis after RFA were the tumor diameter, the number of tumors, the use of combination therapy with ethanol injection, the margin, and the Child-Pugh score.[14] According to the outcome of RFA treatment for a large single-institution series in Korea, the method had a local recurrence rate of 8.1% at 1 year and 11.8% at 3 years, and patient survival rates were 95.2% at 1 year, 69.5% at 3 years, and 58.0% at 5 years.[15] The five year survival after RFA was similar between Western and Eastern countries (Table 1).

Prognosis after RFA and the staging system

The Cancer of the Liver Italian Program (CLIP) score and BCLC scoring system more accurately predicted the prognosis than the Okuda score in patients with early-intermediate HCC, undergoing

Table 1 5-year overall survival after radiofrequency ablation in the patients with operable HCC nodule

			Overall survival	
Investigator	Diameter of the nodule	Patient number	3 y	5y
N'Kontchou G[16]	≤ 5 cm	235		76%
Livraghi T[17]	≤ 2 cm	218		68.5%
Tateichi R[18]	≤ 3 cm	1000	77.7%	54.3%
Peng ZW[19]	≤ 5 cm	281	57.1%	37.1%
	≤ 3 cm		65.7%	58.6%
Choi D[20]		570	69.5%	58.0%

non-surgical therapy, such as RFA.[21] The results of an analysis in Japan demonstrated that, regardless of the CLIP score, the combination of transarterial chemoembolization (TACE)—RFA had the highest 5-year survival.[22] In Japan, where early-stage HCC is prevalent, the majority of cases are classified into CLIP stage 1 of CLIP scores and, as such, the Japanese integrated staging (JIS) score was proposed as a new early HCC staging system.[23] The results of the validation done in many cases demonstrated that the JIS score yielded a better prediction of the prognosis than the CLIP score.[24] It has also been reported from in Korea that the JIS score is the most appropriate score for predicting the prognosis.[25]

Tumor markers

The tumor marker relevant to the prognosis after RFA is des-gamma-carboxy prothrombin time (DCP) levels; wherein, high DCP levels predicted a poor prognosis after RFA.[16,17] However, the same institution also reported that a comparison of AFP, DCP and AFP-leptin 3 (AFP-L3) demonstrated that AFP-L3 was the most useful indicator of the overall survival and disease-free survival.[18] It was pointed out that the AFP mRNA levels in the blood after RFA are also an objective index of recurrence.[19] On the other hand, blood vascular endothelial growth factor (VEGF) levels have also been reported to be related to the prognosis.[20]

Recurrence

Local tumor recurrence after RFA is 9.0% at 1 year and 17.7% at 3 years; therefore, local recurrence is relevant to the prognosis for survival.[26] Evaluation of the therapeutic effects of RFA by contrast enhanced CT scans or by enhanced magnetic resonance imaging (MRI) here demonstrated that the procedure provides good local control and the recurrence rate is low in cases in which the post-ablation margin was 0.4 cm or more and the tumor size was smaller than 2.5 cm.[27] The overall local recurrence rate after RFA was 12.8% and the tumor diameter of >2.5 cm was a significant independent factor.[28] However, another report indicates that even when local recurrence occurred, it did not adversely affect the survival prognosis.[29] Utilizing the RF 3000 generator system has been reported more positive effects than cool-tip electrode.[30]

On the other hand, the cumulative rate of intrahepatic distant recurrence was reported as 10.4% and 77.0% at 1 and 5 years, respectively. In a multivariate analysis, AFP and DCP values, as well as the safety margin, were significant independent factors.[31] The intra-hepatic distant recurrence was associated with multinodular lesions and hepatitis C virus (HCV), even after curative ablation was achieved.[32] Recurrence at a distant site is an important, poor prognostic factor.[33] Although it is possible to ensure long-term survival by carrying out repeat RFA after recurrence,[34] the more frequently recurrences occur, the higher the risk for subsequent recurrence becomes.[35] Histological grade is relevant to the therapeutic efficiency of RFA and also plays a part in determining survival.[36]

Prognosis and possible measures to improve survival after RFA

Long-term interferon maintenance therapy improved the survival in patients with HCV related HCC after RFA.[37] On the other hand, the administration of lamivudine after RFA for hepatitis B virus (HBV)-related HCC maintained the liver function and was also safe.[38,39] The administration of vitamin K for HCV-related HCC did not produce a chmenopreventative effect.[40] The oral administration of a branched-chain amino acid after RFA made it possible to maintain the serum albumin levels and it was also useful for improving the liver function.[41]

Resection *versus* RFA

With regard to the question of whether surgical resection or RFA is superior, two randomized comparisons have been reported—all from China. In these reports, the life prognoses of single HCCs of 2 cm or less diameter were randomly compared between RFA and resection. It was reported that there would be no difference between the two, or that, for single HCC of 5 cm or less, there was no difference in terms of both disease-free survival and overall survival.[42,43] In Italy, a group of 109 patients who underwent RFA and a group of 91 patients who underwent resection were compared retrospectively; there was no difference in terms of the overall survival and disease-free survival, for HCC of 3 cm or less.[44] Likewise, a retrospective analysis conducted in Korea, compared a group of 55 patients who underwent RFA treatment for single HCC 4 cm or less and well-preserved liver function with a group of 93 patients who underwent resection; the authors concluded that there was no difference in terms of overall survival and recurrence-free survival at 1 year and 3 years after RFA.[45] When laparoscopic RFA was performed on patients with single HCC nodule with Child-Pugh A liver function, RFA and resection had similar survival rates.[46,47]

However, a case control study of resection versus RFA showed that recurrence, tumor diameter, and whether resection or RFA were performed, all affected overall survival. The authors con-

cluded that a resection provided some advantages.[48] Furthermore, with regard to cases of HCC which are not suitable candidates for liver transplantation, a Markov model was used to compare the life-adjusted survival between resection and RFA. The survival rate in the resection group was 5.33 years, while in the RFA group it was 3.91 years. It was concluded therefore, that patients treated by a resection would have a better survival rates.[49] In another study, 79 cases of resection and 79 cases of RFA treated at two different institutions were compared. The result showed that resection would be better than RFA for tumors of 3 cm and larger in diameter with Child A score, but that the overall survival would be the same for surgery and RFA in the case of Child B score.[50]

Comparison between RFA and other ablations

A comparison between microwave coagulation and RFA for HCC, 2 cm or less in tumor diameter demonstrated that RFA was superior because it created a larger necrotic area, resulting in a lower local recurrence rate; this conferred better cumulative survival, while bile duct injury and pleural effusion occurred less frequently.[51]

Another study compared percutaneous ethanol injection (PEI) and RFA. This randomized controlled trial (RCT) conducted in Taiwan demonstrated that RFA required fewer treatment sessions to achieve complete tumor necrosis, and provided better overall survival.[52] Another RCT between PEI and RFA was conducted in Japan. The 4-year survival rates were 74% for RFA versus 57% for PEI, resulting in RFA treatment being associated with a lower risk of death and recurrence. There was no difference in frequency of adverse events.[53] Although it was not RCT, another study compared PEI and RFA and found that local recurrence rates after RFA were lower.[54] An RCT conducted in Italy compared RFA with PEI and found that complete response of RFA after one year was associated with a better outcome, though no survival advantage was observed.[55]

There have been three meta-analyses, based on RCT comparing the effects and complications between RFA and PEI. Each found that RFA had better overall survival, while PEI had a higher local recurrence rate; thus RFA was superior in cancer-free survival rates.[56–58] No difference was observed in the complications between the two.

RCT was conducted to identify whether a combination of RFA and PEI would produce a better outcome than RFA alone. For tumors measuring between 3.1 cm and 5 cm in size, RFA + PEI improved patient survival, and overall recurrence was lower with combination treatment.[59]

Combined TACE and RFA treatment

The combination of transarterial embolization (TAE) and RFA or PEI was compared with TACE alone, and it was found that TACE + RFA had a better prognosis.[60] The results of a case-control comparison between RFA combined with TACE and RFA alone demonstrated that there was no difference in cases of single HCC $\leq$ 5 cm, but that the TACE + RFA combined treatment had a higher survival rate in cases of single HCC > 5 cm or multiple tumors.[61] The combination of TACE and RFA was technically successful in 88% of cases; such patients, complete the therapy after a single treatment session.[62] In addition, the combination of TACE and RFA produced high local control rates.[63] TACE and RFA has been performed for HCC immediately below the diaphragm, and found to be effective.[64] The combination of bland arterial embolization with RFA and a resection has also been compared; the overall survival was found to be similar in patients with single HCC measuring up to 7 cm in diameter.[65]

The extent of necrosis resulting from RFA increases when combined with hepatic arterial balloon occlusion.[66] Furthermore, combined treatment with balloon occlusion after transcatheter arterial infusion chemotherapy (TAI) is effective in expanding the necrotic area.[67] However, some researchers argue that this combination is not necessary because the effects of the combined therapy involving TACE and RFA, and that of RFA alone, for small HCC $\leq$ 3 cm, are the same.[68]

Complications

Data from 3891 cases were collected in a joint study conducted in Osaka, Japan. Complications were observed in 207 cases (7.9%), with 9 patients dying within 3 months. The causes of death in these cases were: liver failure in 3 cases, rapid progression in 3 cases, biliary injury in 1 case, gastrointestinal bleeding in 1 case, and myocardial infarction in 1 case.[69] Data for 255 cases in China have also been reported, with major complications observed in 31 cases (10%) as follows: 13 cases of liver failure, 10 cases of hydrothorax, 2 cases of tumor seeding, 1 case of upper gastrointestinal bleeding, and one each of intrahepatic abscess, bile duct injury, and cardiac arrest, 5 cases of hyperglycemia, and 11 cases of death due to liver failure.[70] A report from the United States noted that complications had been observed in 7 out of 91 cases as follows: 2 cases of hepatic abscess and one each of skin burn, hemorrhage, myocardial infarction, and liver failure.[71] According to the results of a multicenter survey conducted in Korea, liver abscess (0.66%), peritoneal hemorrhage (0.46%), biloma (0.20%), ground pad burn (0.20%) and pneumothorax (0.20%) were reported as complications.[72]

Liver abscess and bile duct injury

Liver abscess is the most common complication—de Raere *et al.* observed 7 cases out of 350 sessions and a high risk of this complication among patients with a revious bilioenteroc anasomosis.[73] Likewise, Choi *et al.*[74] and Elias *et al.*[75] also reported that liver abscess was seen more often in cases of biliary abnormality, as well as after TACE treatment. In one report, cholangitis and liver abscess occurred simultaneously.[76] Attention should therefore be paid to the fact that the risk for liver abscess complication is high in cases of complicated anastomosis of the bile duct to the intestinal tract.

Biliary stricture was observed in 7 cases after the RFA procedure, with liver abscess as a complication in 3 cases.[77] It was reported that intraductal chilled saline perfusion by endoscope had been effective in preventing bile duct injury.[78]

Bleeding

A total of 4133 RFA treatments were performed in 2154 cases, with hemorrhagic complications occurring in 63 treatments (1.5%)

as follows: hemoperitoneum (0.7%), hemothorax (0.3%) and hemobilia (0.5%). In addition, there were two deaths due to hemoperitoneum.[79] Poggi *et al.* reported only one case of bleeding that required surgery.[80] Attention has also been focused on bleeding which occurred in one case of subcapsular liver tumor, but there were no complications such as seeding.[81,82]

Intestinal injury

Two cases were reported in which colonic perforation occurred as a complication on the 8th day after RFA. Attention should be paid to the fact that intestinal injury was indolently present.[83,84] Another report indicated the occurrence of duodenopleural fistula formation as a complication.[85]

Hepatic infarction

Hepatic infarction has been observed after RFA; the frequency is 1.8%.[86] The use of internally cooled electrodes is a risk. In addition, portal thrombosis has also been reported to occur.[73,87]

Seeding

A report given by Llovet *et al.* in 2001 on the high rate of seeding after RFA has received much attention. The risk factors included: subcapsular tumor localization, a high degree of poor differentiation, and a high baseline AFP.[88] Since then the risk of seeding after RFA, has been attributed to subcapsular location, poorly differentiated tumors and high AFP levels.[89,90] However, a discrepancy exists between institutions, with some arguing that, in reality, seeding is exceptionally rare.[91,92] In order to prevent seeding, tract ablation should therefore be properly performed.

Other complications

In another report, pneumothorax occurred after RFA, and so careful attention is required for tumors adjacent to the diaphragm.[93] It has been reported that myoglobinuria occurs as a complication after RFA and that the serum creatinin level rises, making it necessary for attention to be paid thereto.[94] Another case was reported in which hemolysis occurred, thus inducing hemogeobinuria as a complication.[95] There have also been reports that rapid tumor progression occurred after RFA;[96,97] however, the actual frequency was low and it is therefore necessary to investigate whether or not it was indeed a complication associated with RFA.

We have done 1440 sessions of RFA to patients with early stage HCC from July, 1999 to December 2009. The complications have been analyzed as shown in Table 2. The complication rates were 1.8% and 1.9% when the patients were treated by laparoscopic or percutaneous RFA, respectively.

Conclusion

RFA is promising for improving patient survival with early stage of HCC when performed skillfully to avoid serious complication. To prevent the recurrence of HCC is the most important issue for achieving better survival.

Table 2 Complications by laparoscopic or percutaneous RFA in Musashino Red-Cross hospital (from July, 1999 to December, 2009)

	Laparoscopic RFA (n = 107)	Percutaneous RFA (n = 1333)
Bile duct damage	0 (%)	4 (0.4%)
Liver abscess	1 (0.9%)	4 (0.3%)
Inter-costal arterial injury	0 (0%)	3 (0.3%)
Hemothorax	0 (0%)	4 (0.3%)
Hepatic infarction	0 (0%)	3 (0.3%)
Hepatic dysfunction	0 (0%)	2 (0.3%)
Skin burn	0 (0%)	2 (0.2%)
Subcutaneous hematoma	1 (0.9%)	0 (0%)
Peumothorax	0 (0%)	2 (0.1%)
Gastrointestinal perforation	0 (0)	1 (0.1%)
Total	2 (1.8%)	26 (1.9%)

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