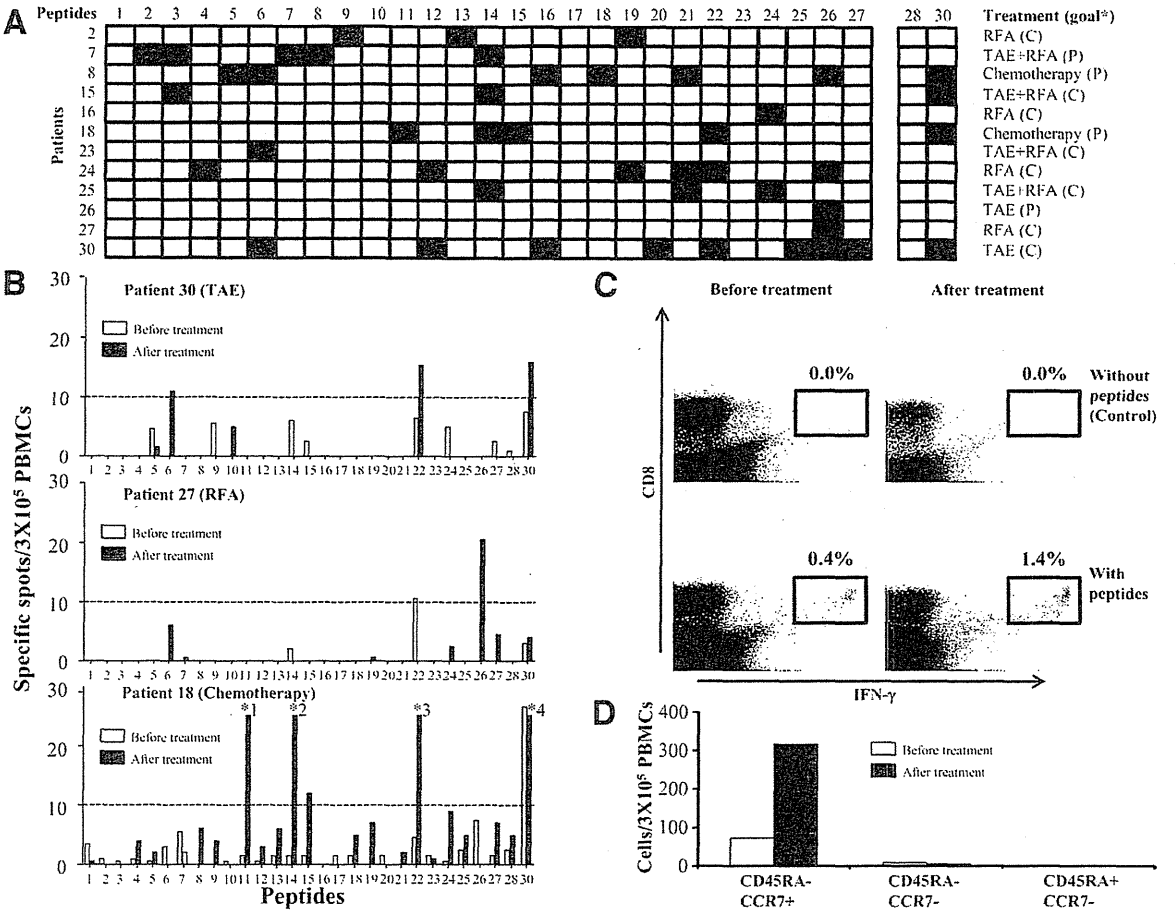

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-

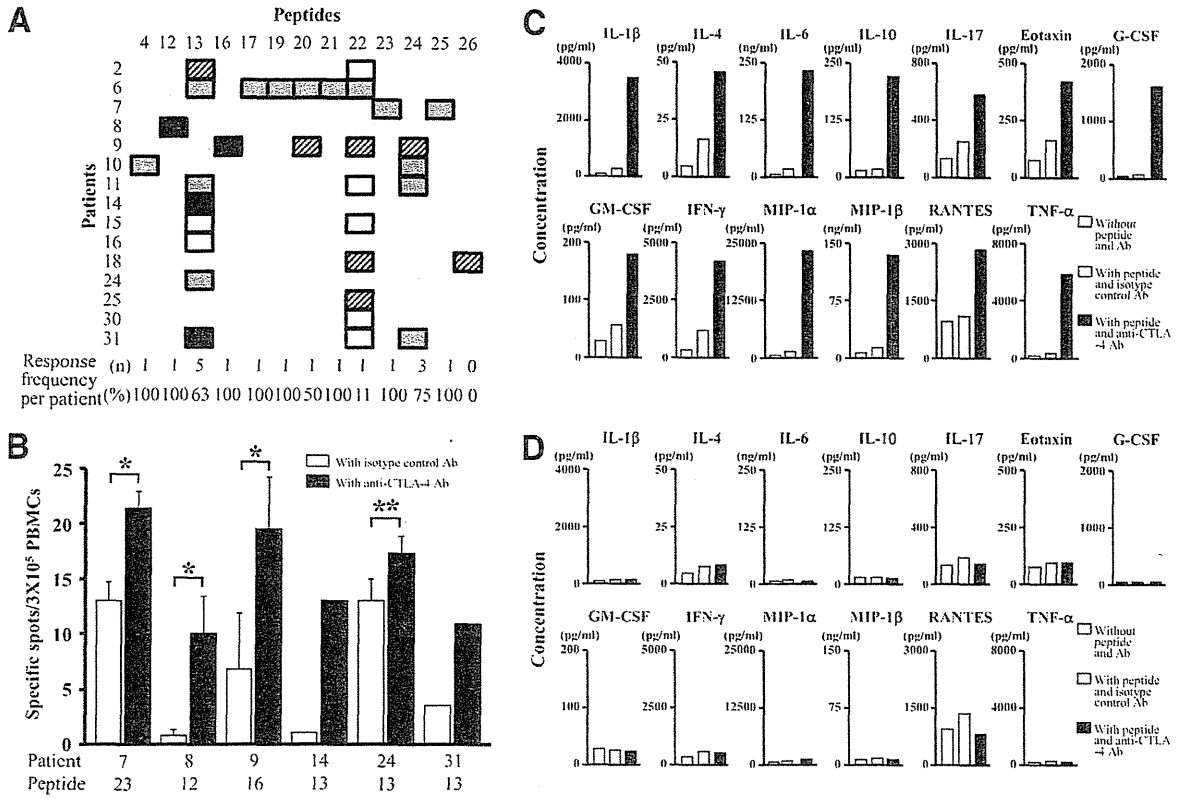

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^-/CCR7^+$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.

On the other hand, recent studies have shown the important role of $CD4^+$ helper T cells in optimal function and proliferation of $CD8^+$ T cells.[46] Therefore, the lack of $CD4^+$ helper T cells or anergic $CD4^+$ T cells may explain the limited TAA-specific $CD8^+$ T-cell responses in HCC. Further studies using $CD4^+$ T-cell-depleted PBMCs or $CD8^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

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References

1. Deuffic S, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. Lancet 1998;351:214-215.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153-156.
3. Lencioni R. Loco-regional treatment of hepatocellular carcinoma. HEPATOLOGY 2010;52:762-773.
4. Okuwaki Y, Nakazawa T, Shibuya A, Ono K, Hidaka H, Watanabe M, et al. Intrahepatic distant recurrence after radiofrequency ablation for a single small hepatocellular carcinoma: risk factors and patterns. J Gastroenterol 2008;43:71-78.
5. Nishizaka S, Gomi S, Harada K, Oizumi K, Itoh K, Shichijo S. A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma. Cancer Res 2000;60:4830-4837.
6. Kawano K, Gomi S, Tanaka K, Tsuda N, Kamura T, Itoh K, et al. Identification of a new endoplasmic reticulum-resident protein recognized by HLA-A24-restricted tumor-infiltrating lymphocytes of lung cancer. Cancer Res 2000;60:3550-3558.
7. Gomi S, Nakao M, Niiya F, Imamura Y, Kawano K, Nishizaka S, et al. A cyclophilin B gene encodes antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs. J Immunol 1999;163:4994-5004.
8. Harashima N, Tanaka K, Sasatomi T, Shimizu K, Miyagi Y, Yamada A, et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. Eur J Immunol 2001;31:323-332.
9. Fujie T, Tahara K, Tanaka F, Mori M, Takesako K, Akiyoshi T. A MAGE-1-encoded HLA-A24-binding synthetic peptide induces specific anti-tumor cytotoxic T lymphocytes. Int J Cancer 1999;80:169-172.
10. Nishiyama T, Tachibana M, Horiguchi Y, Nakamura K, Ikeda Y, Takesako K, et al. Immunotherapy of bladder cancer using autologous dendritic cells pulsed with human lymphocyte antigen-A24-specific MAGE-3 peptide. Clin Cancer Res 2001;7:23-31.
11. Kikuchi M, Nakao M, Inoue Y, Matsunaga K, Shichijo S, Yamana H, et al. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. Int J Cancer 1999;81:459-466.
12. Nakao M, Shichijo S, Imaizumi T, Inoue Y, Matsunaga K, Yamada A, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. J Immunol 2000;164:2565-2574.
13. Yang D, Nakao M, Shichijo S, Sasatomi T, Takasu H, Matsumoto H, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. Cancer Res 1999;59:4056-4063.
14. Tanaka H, Tsunoda T, Nukaya I, Sette A, Matsuda K, Umano Y, et al. Mapping the HLA-A24-restricted T-cell epitope peptide from a tumour-associated antigen HER2/neu: possible immunotherapy for colorectal carcinomas. Br J Cancer 2001;84:94-99.
15. Eura M, Chikamatsu K, Katsura F, Obata A, Sobao Y, Takiguchi M, et al. A wild-type sequence p53 peptide presented by HLA-A24 induces cytotoxic T lymphocytes that recognize squamous cell carcinomas of the head and neck. Clin Cancer Res 2000;6:979-986.
16. Umano Y, Tsunoda T, Tanaka H, Matsuda K, Yamaue H, Tanimura H. Generation of cytotoxic T-cell responses to an HLA-A24 restricted epitope peptide derived from wild-type p53. Br J Cancer 2001;84:1052-1057.
17. Ferries E, Connan F, Pages F, Gaston J, Hagnere AM, Vieillefond A, et al. Identification of p53 peptides recognized by CD8(+) T lymphocytes from patients with bladder cancer. Hum Immunol 2001;62:791-798.
18. Yamada A, Kawano K, Koga M, Matsumoto T, Itoh K. Multidrug resistance-associated protein 3 is a tumor rejection antigen recognized by HLA-A2402-restricted cytotoxic T lymphocytes. Cancer Res 2001;61:6459-6466.
19. Mizukoshi E, Nakamoto Y, Tsuji H, Yamashita T, Kaneko S. Identification of alpha-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma. Int J Cancer 2006;118:1194-1204.
20. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, et al. Cytotoxic T-cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. HEPATOLOGY 2006;43:1284-1294.
21. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415-2432.
22. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321-327.
23. Itoh K, Yamada A. Personalized peptide vaccines: a new therapeutic modality for cancer. Cancer Sci 2006;97:970-976.
24. Butterfield LH, Ribas A, Meng WS, Dissette VB, Amarnani S, Vu HT, et al. T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 2003;9:5902-5908.
25. Butterfield LH, Ribas A, Dissette VB, Lee Y, Yang JQ, De la Rocha P, et al. A phase I/II trial testing immunization of hepatocellular carcinoma patients with dendritic cells pulsed with four alpha-fetoprotein peptides. Clin Cancer Res 2006;12:2817-2825.
26. Butterfield LH. Recent advances in immunotherapy for hepatocellular cancer. Swiss Med Wkly 2007;137:83-90.
27. Dougan M, Dranoff G. Immune therapy for cancer. Annu Rev Immunol 2009;27:83-117.
28. O'Day SJ, Maio M, Chiarion-Sileni V, Gajewski TF, Pehamberger H, Bondarenko IN, et al. Efficacy and safety of ipilimumab monotherapy in patients with pretreated advanced melanoma: a multicenter single-arm phase II study. Ann Oncol 2010;21:1712-1717.
29. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037-1043.

30. Japan. LCSGo. Classification of primary liver cancer. English ed 2. Tokyo: Kanehara; 1997.
31. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
32. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, et al. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242-6252.
33. Kurokohchi K, Arima K, Nishioka M. A novel cytotoxic T-cell epitope presented by HLA-A24 molecule in hepatitis C virus infection. J Hepatol 2001;34:930-935.
34. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 2001;98:1872-1881.
35. Oiso M, Eura M, Katsura F, Takiguchi M, Sobao Y, Masuyama K, et al. A newly identified MAGE-3-derived epitope recognized by HLA-A24-restricted cytotoxic T lymphocytes. Int J Cancer 1999;81:387-394.
36. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000;192:303-310.
37. Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, et al. Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. Cancer Res 2006;66:1139-1146.
38. Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, et al. Unmasking of alpha-fetoprotein-specific CD4(+) T-cell responses in hepatocellular carcinoma patients undergoing embolization. J Immunol 2007;178:1914-1922.
39. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708-712.
40. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. J Exp Med 2009;206:1717-1725.
41. Zerbini A, Pilli M, Soliani P, Ziegler S, Pelosi G, Orlandini A, et al. Ex vivo characterization of tumor-derived melanoma antigen encoding gene-specific CD8+cells in patients with hepatocellular carcinoma. J Hepatol 2004;40:102-109.
42. Gehring AJ, Ho ZZ, Tan AT, Aung MO, Lee KH, Tan KC, et al. Profile of tumor antigen-specific CD8 T cells in patients with hepatitis B virus-related hepatocellular carcinoma. Gastroenterology 2009;137:682-690.
43. Fujioka M, Nakashima Y, Nakashima O, Kojiro M. Immunohistologic study on the expressions of alpha-fetoprotein and protein induced by vitamin K absence or antagonist II in surgically resected small hepatocellular carcinoma. HEPATOLOGY 2001;34:1128-1134.
44. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 2007;26:2166-2176.
45. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology 2007;132:2328-2339.
46. Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. Immunol Rev 2008;222:129-144.

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Randomized, Phase II Study Comparing Interferon Combined with Hepatic Arterial Infusion of Fluorouracil plus Cisplatin and Fluorouracil Alone in Patients with Advanced Hepatocellular Carcinoma

Tatsuya Yamashita[a] Kuniaki Arai[a] Hajime Sunagozaka[a] Teruyuki Ueda[a]
Takeshi Terashima[a] Taro Yamashita[a] Eishiro Mizukoshi[a] Akito Sakai[a]
Yasunari Nakamoto[b] Masao Honda[a] Shuichi Kaneko[a]

[a]Department of Gastroenterology, Kanazawa University, Kanazawa, and [b]Department of Gastroenterology, Fukui University, Fukui, Japan

Key Words
Hepatic arterial infusion · Hepatocellular carcinoma · 5-Fluorouracil · Cisplatin · Interferon

Abstract
Objective: This randomized phase II trial compared the response rates to treatment with interferon (IFN) combined with hepatic arterial infusion of fluorouracil (FU) plus cisplatin (CDDP) or FU alone in patients with advanced hepatocellular carcinoma (HCC). ***Methods:*** A total of 114 patients with measurable advanced HCC were enrolled and randomized into 2 groups. FU (300 mg/m^2, days 1–5, days 8–12) with or without CDDP (20 mg/m^2, days 1 and 8) was administered via the hepatic artery. IFNα-2b was administered 3 times per week for 4 weeks. ***Results:*** The response rates were 45.6% for the IFN/FU + CDDP group and 24.6% for the IFN/FU group. The response rate was significantly higher in the IFN/FU + CDDP group ($p = 0.030$). The median overall survival period was 17.6 months in the IFN/FU + CDDP group versus 10.5 months in the IFN/FU group ($p = 0.522$). The median progression-free survival period was 6.5 months in the IFN/FU + CDDP group versus 3.3 months in the IFN/FU group ($p = 0.0048$). Hematological toxicity was common, but no toxicity-related deaths were observed. ***Conclusion:*** These results show the clinical efficacy of adding CDDP to the hepatic arterial infusion of FU in combined chemotherapy regimens with IFN.

Introduction

Hepatocellular carcinoma (HCC) is the 6th most frequent type of cancer in the world and ranks third among the various causes of cancer death. In recent years, the incidence of HCC has been increasing in Western and Asian countries [1–3].

Clinical practice guidelines for HCC are currently available in Japan, and the number of early cases with an early single tumor with a major diameter of 2 cm or less detected by regular screening is generally increasing [4]. The treatment of early cases, including the use of hepatectomy and local therapy such as radiofrequency abla-

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Tatsuya Yamashita
Department of Gastroenterology, Kanazawa University
Takara-machi 13-1
Kanazawa, Ishikawa 920-8641 (Japan)
Tel. +81 76 265 2861, E-Mail ytatsuya@m-kanazawa.jp

tion and percutaneous ethanol injection therapy, has progressed markedly, achieving a 5-year survival rate of 60–70% [5]. Most patients with HCC experience the repeated recurrence of tumors after treatment, and the disease may eventually reach an advanced stage. Furthermore, it is still not uncommon to find patients with symptomatic advanced HCC who have not participated in regular screening.

The efficacy of hepatectomy, local ablation therapy and transarterial chemoembolization (TACE) is limited for advanced HCC, and the prognosis of such cases is poor. Under these circumstances, systemic therapy with the molecular targeting drug sorafenib has shown a statistically significant survival benefit compared with placebo treatment in two large-scale phase III clinical trials [6, 7]. Based on these findings, this drug is now recommended as a standard treatment for advanced HCC. These trials did not compare sorafenib with other conventional treatments of advanced HCC but with best supportive care as the placebo treatment. Although a significant difference in the survival time was noted, the response rate was as low as 2–3.3%, with no significant difference from the results in the placebo arm (1–1.3%) [6, 7].

As another optional treatment for advanced HCC, hepatic arterial infusion chemotherapy (HAIC) has been employed mainly in Japan and other Asian countries. HAIC has been used not only for unresectable HCC accompanied by vascular invasion but also uncontrollable cases of repeated recurrences within a short period of time despite a number of sessions of TACE.

In recent years, fluorouracil (FU) and cisplatin (CDDP) have been reported as the most commonly used anticancer drugs for HAIC [8–17]. Favorable results with an HAIC protocol using low-dose CDDP and FU have also been reported [8, 14, 16, 17]. Similarly, combination of interferon (IFN) with FU has demonstrated relatively good results in HAIC [11, 13, 18].

With this background in mind and with the aim of establishing the most effective HAIC protocol for advanced HCC, we planned a phase II randomized clinical comparative study to examine whether or not IFN combined with HAIC consisting of FU and CDDP might be associated with a higher response rate. Patients with advanced HCC were randomly allocated to two treatment arms, i.e. IFN combined with hepatic arterial infusion of FU with CDDP or IFN combined with hepatic arterial infusion of FU alone without CDDP. The results were then compared with regard to the efficacy, safety and prognosis.

Patients and Methods

Patients

Patients who had histologically or clinically diagnosed HCC were included in this study. A clinical diagnosis of HCC was made based on underlying chronic liver disease, radiologic findings and elevation of tumor markers.

With regard to the tumor stage, the following patients were included: patients who had (1) severe vascular invasion (i.e. vascular invasion found in the main trunk to the secondary branches of the portal vein, or invasion in the right, middle or left hepatic vein) and (2) intrahepatic multiple lesions (i.e. 5 or more nodules in the left and/or right lobes as confirmed by radiology).

Patients were eligible when they were 20 years old or older, had an Eastern Clinical Oncology Group performance status of 2 or less and had appropriate bone marrow, liver, kidney and cardiac functions as determined by the following measurements obtained within 1 week before enrollment: hemoglobin ≥8.0 g/dl; white blood cell count ≥2,000/mm^3; platelet count ≥30,000/mm^3; blood urea nitrogen ≤30 mg/dl; serum creatinine ≤2.0 mg/dl; percentage of prothrombin time ≥30%, and total bilirubin ≤5 mg/dl or less (excluding elevations caused by biliary tract obstruction as a result of HCC).

Assignment

The present study was an open randomized single-center study consisting of a two-group comparison. All the patients who satisfied the inclusion criteria were randomized to either of the two treatments. The treatment protocol was approved by the ethical committee of Kanazawa University (approval number 5169). Patients were given full information regarding the details of the clinical study and provided their written consent prior to participation in the study. This clinical study adhered to the Declaration of Helsinki and good clinical practice.

Treatment Schedule

A reservoir for hepatic arterial infusion was implanted prior to HAIC. A catheter with a side hole was inserted from the right femoral artery using an image-guided procedure, and the tip of the catheter was placed in the gastroduodenal artery or splenic artery. When more than one hepatic artery was present, the hepatic arteries were unified to the original proper hepatic artery alone. When blood flow into the gastrointestinal tract was confirmed by catheter angiography, the route was embolized to prevent complications. The reservoir was placed beneath the skin in the lower right abdomen. Medication was started at least 3 days after implantation.

In the IFN/FU treatment group, patients underwent continuous hepatic arterial infusion of FU (5-FU®, Kyowa Hakko, Tokyo, Japan) at a dose of 300 mg/m^2/day for 5 days in the 1st and 2nd weeks (for 120 h) using an infuser pump (Baxter Infusor SV1®, Tokyo, Japan) in the same manner as described in previous reports [18]. The maximum amount of FU infused over 5 days was 2,500 mg. IFNα-2b (Intron A®, Schering-Plough, Osaka, Japan) at a dose of 3,000,000 units was injected intramuscularly 3 times a week for 4 weeks. In the IFN/FU + CDDP treatment group, CDDP (Randa®, Nippon Kayaku, Tokyo, Japan) at a dose of 20 mg/m^2 was given by hepatic arterial infusion over 1.5 h on days 1 and 8 prior to the administration of FU and after appropriate hy-

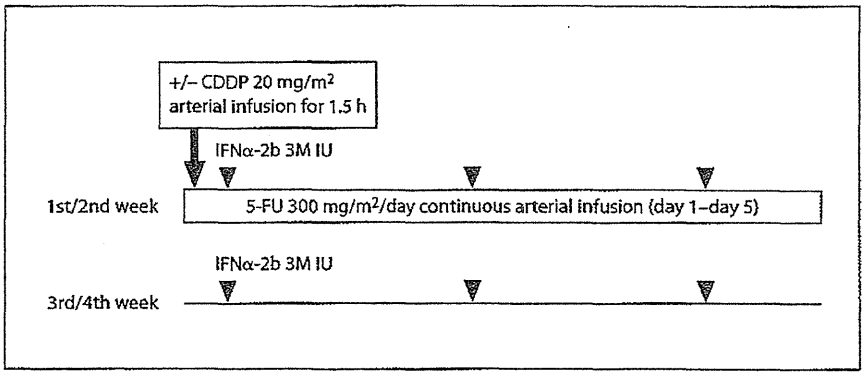

Fig. 1. Treatment protocol. 3M = 3 million.

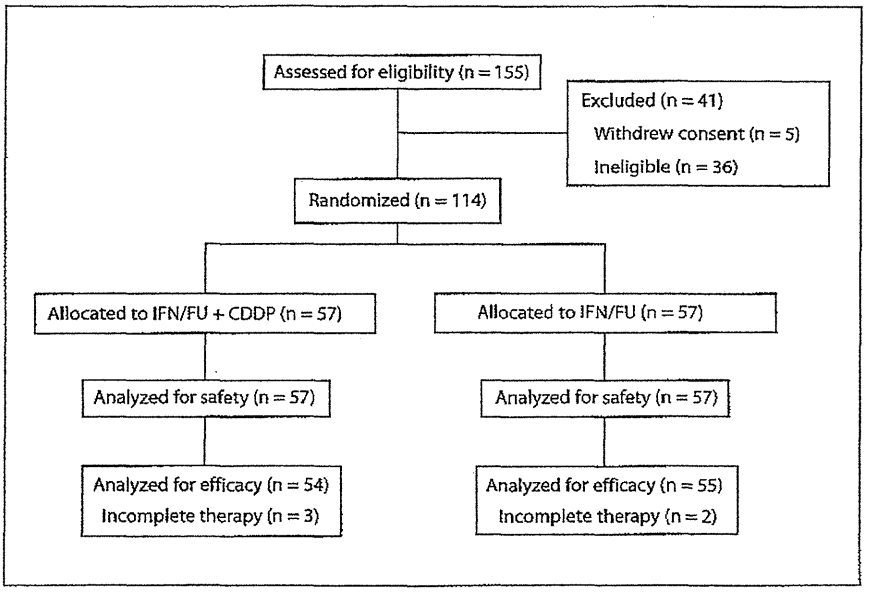

Fig. 2. Consolidated Standards of Reporting Trials flow diagram.

dration and antiemetic medication. A treatment cycle comprised 4 weeks of drug administration including IFN administration and a subsequent 2-week rest period (fig. 1).

Sample Size

Based on previous reports in the literature [9, 19] and the results of our studies of HAIC for the treatment of HCC using single-drug regimens, it was assumed that the response rate in the IFN/FU treatment group would be 20% and that in the IFN/FU + CDDP treatment group would be 50%. Based on the assumption that the ratio of the numbers of patients was 1:1, the α error was 0.05, the β error was 0.1, and 52 patients were necessary for each treatment group. Therefore, the number of patients to be included was 114, allowing a 10% dropout rate, which would result in a total of 104 patients for the two groups.

Response Assessment

The primary endpoint was the response rate, as determined using dynamic computed tomography or magnetic resonance imaging performed at the end of each treatment cycle according to the Response Evaluation Criteria in Solid Tumors, version 1.0 [20].

Secondary endpoints were the overall survival time, progression-free survival time and adverse events. The overall survival time was defined as the period from the time of randomization until death, and the progression-free survival time was defined as the period from the beginning of treatment until confirmation of progression or death. Adverse events were evaluated according to the Common Toxicity Criteria for Adverse Events, version 3.0.

Statistical Analyses

The two treatment groups were compared using the Fisher direct method and the Wilcoxon rank sum test. Response factors were analyzed using logistic regression analysis. The cumulative survival and prognostic factors were analyzed using the Kaplan-Meier method, log-rank test and the Cox proportional hazard regression model.

Table 1. Patient demographics and baseline characteristics

	IFN/FU + CDDP (n = 57)	IFN/FU (n = 57)	p value
Gender (male/female)	49/8	46/11	0.62[a]
Median age, years (range)	65 (40–82)	68 (40–82)	0.27[b]
ECOG PS (0/1/2)	36/19/2	34/21/2	0.92[b]
Primary/recurrence	20/37	23/34	0.70[a]
Prior TACE (+/–)	32/25	33/24	1.00[a]
Prior chemotherapy (+/–)	4/53	3/54	1.00[a]
HCV-Ab (positive/negative)	32/25	35/22	0.70[a]
HBsAg (positive/negative)	16/41	18/39	0.83[a]
Liver cirrhosis (+/–)	46/11	47/10	1.00[a]
Child-Pugh class (A/B/C)	33/23/1	32/22/3	0.74[b]
LCSGJ TNM stage (II/III/IVA/IVB)	7/26/17/7	7/20/25/5	0.53[b]
UICC TNM stage (II/III/IV)	6/43/8	12/38/7	0.30[b]
BCLC stage (B/C/D)	33/23/1	23/31/3	0.13[b]
Median diameter of tumor, mm (range)	37 (10–250)	40 (11–200)	0.71[b]
Major portal vein invasion (+/–)	12/45	19/38	0.21[a]
Lymph node metastasis (+/–)	2/55	4/53	0.68[a]
Distant metastasis (+/–)	7/50	5/52	0.76[a]
Treatment cycles, n	3.2 ± 2.6	2.9 ± 2.4	0.37[b]
Albumin, g/dl	3.36 ± 0.6	3.49 ± 0.5	0.22[b]
Total bilirubin, mg/dl	1.10 ± 0.7	1.44 ± 0.88	0.07[b]
Active prothrombin, %	78.6 ± 18.9	74.9 ± 13.8	0.22[b]
Platelet count, $\times 10^4/\mu l$	12.3 ± 6.4	11.0 ± 5.4	0.26[b]
AST, IU/l	83.1 ± 74.4	82.5 ± 51.8	0.47[b]
ALT, IU/l	64.2 ± 53.6	68.5 ± 88.6	1.00[b]
DCP (<100/≥100 mAU/ml)	33/24	37/20	0.56[a]
AFP (<400/≥400 ng/ml)	24/33	28/29	0.57[a]
AFP-L3 (<30/≥30%)	22/35	27/30	0.45[a]

Values represent numbers of patients or mean ± SD, except where indicated otherwise. ECOG = Eastern Cooperative Oncology Group; PS = performance status; HBsAg = hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; UICC = Union for International Cancer Control; BCLC = Barcelona Clinic Liver Cancer; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein.
[a] Fisher's exact test. [b] Wilcoxon rank sum test.

Results

Patients

A total of 155 patients with advanced HCC were treated at our hospital between October 2003 and September 2007. Eventually, 114 patients were allocated to the IFN/FU + CDDP treatment group or the IFN/FU treatment group. Three patients in the IFN/FU + CDDP group and 2 in the IFN/FU group dropped out before the end of the first cycle; therefore, a total of 109 patients, comprising 54 patients from the former group and 55 from the latter, were included in the efficacy evaluation (fig. 2).

The baseline clinical features of the 114 patients are shown in table 1. No significant differences in the clinical features and test results were observed between the two groups, with the exception of a slightly higher bilirubin level in the IFN/FU group. The patients classified as having Barcelona Clinic Liver Cancer stage B had 5 or more nodules in the left and/or right lobes and were considered to have disease that was difficult to control by TACE after repeated TACE (68%) or multiple lesions that showed an inadequate response to TACE.

Response to Treatment

Among the 57 patients in the IFN/FU + CDDP treatment group, the best study response was a complete response (CR) in 1 (1.7%); partial response (PR) was observed in 25 (43.9%) patients, stable disease was observed

Table 2. Comparison of best study response between treatment arms

Best study response	IFN/FU + CDDP (n = 57)	IFN/FU (n = 57)	p value
CR	1 (1.7)	3 (5.3)	
PR	25 (43.9)	11 (19.3)	
SD	15 (26.3)	19 (33.3)	
PD	13 (22.8)	22 (38.6)	
NE	3 (5.3)	2 (3.5)	
RR (CR + PR)	26 (45.6)	14 (24.6)	0.030
TCR (CR + PR + SD)	41 (71.9)	33 (57.9)	0.169

Values represent n (%). Between-group p values were determined using Fisher's exact test. SD = Stable disease; PD = progressive disease; NE = not evaluable; RR = response rate; TCR = tumor control rate.

in 15 (26.3%), and progressive disease was observed in 13 (22.8%). Among the 57 patients in the IFN/FU treatment group, the response was CR in 3 (5.3%), PR in 11 (19.3%), stable disease in 19 (33.3%) and progressive disease in 22 (38.6%). The response rate (CR + PR) was 45.6% in the IFN/FU + CDDP group and 24.6% in the IFN/FU group; the figure was significantly higher in the former group (p = 0.030; table 2).

The only factor that improved the response to treatment as indicated by a multivariate analysis was the addition of CDDP to the treatment [odds ratio 2.5, 95% confidence interval (CI) 1.1–6.0; table 3].

Safety

Table 4 shows the major adverse events. Grade 3 or 4 adverse events were found in 75 of the 114 patients (65.8%). Bone marrow suppression of any grade was found in 65–90% of the patients. Leukopenia and neutropenia were noted in about 70% of the patients, and no significant difference was found between the IFN/FU + CDDP group and the IFN/FU group. An overall reduction in hemoglobin was observed more frequently in the IFN/FU + CDDP group than in the IFN/FU group (91.2 vs. 75.4%; p = 0.021), although the difference was not significant for hemoglobin reductions of grade 3 or 4. No significant difference in all-grade thrombocytopenia was observed between the two groups, but thrombocytopenia of grade 3 or 4 was found significantly more frequently in the IFN/FU + CDDP group (45.6 vs. 22.8%; p = 0.017). However, no serious complications secondary to a reduction in platelets occurred.

Nonhematologic toxicities including general malaise, nausea, vomiting, stomatitis and elevation of serum creatinine were significantly more common in the IFN/FU + CDDP group, but no intergroup difference was found for grade 3 or grade 4 toxicities.

Peptic ulcer arising from the leakage of arterially infused anticancer drugs into the gastrointestinal tract, a complication characteristic of HAIC, was found in 6 patients (10.5%) in the IFN/FU + CDDP group and 1 patient (1.8%) in the IFN/FU group; the incidence was higher, but not significantly, in the IFN/FU + CDDP group (p = 0.06), and no grade 3 or grade 4 cases occurred.

Survival

The median overall survival period of the 114 patients who underwent HAIC was 12.0 months (95% CI 11.6–12.4). In the IFN/FU + CDDP group, the median survival time (MST) was 17.6 months (95% CI 9.9–25.3). In the IFN/FU group, the MST was 10.5 months (95% CI 5.6–15.4). Although the survival period tended to be longer in the group given FU combined with CDDP, no statistically significant differences were observed between the two groups (p = 0.522, log-rank test; hazard ratio 0.88, 95% CI 0.60–1.30; fig. 3a).

In the subgroup with the presence of major vascular invasion, the MST was 5.8 months (95% CI 3.3–8.3) in the IFN/FU + CDDP group and 4.7 months (95% CI –7.6 to 31.6) in the IFN/FU group. In contrast, in the subgroup with absence of major vascular invasion, the MST was 20.0 months (95% CI 13.6–26.6) in the IFN/FU + CDDP group and 12.0 months (95% CI 4.4–19.6) in the IFN/FU group. Subanalysis according to the presence or absence of major vascular invasion showed no significant difference between the two treatment groups (p = 0.571 in the presence of major vascular invasion, p = 0.399 in its absence). In the subgroup with tumor stage II and III, the MST was 22.6 months (95% CI 0.4–44.7) in the IFN/FU + CDDP group and 12.0 months (95% CI 5.5–18.5) in the IFN/FU group. In the subgroup with tumor stage IVA and IVB, the MST was 7.5 months (95% CI 5.7–9.3) in the IFN/FU + CDDP group and 7.5 months (95% CI 0.4–14.5) in the IFN/FU group. Subanalysis according to tumor stage (stage II and III or stage IVA and IVB) also showed no difference between the two treatment groups (p = 0.625 for stage II and III, p = 0.906 for stage IVA and IVB).

The median overall progression-free survival period of the 114 patients was 4.5 months (95% CI 3.5–5.5). In the IFN/FU + CDDP group, the median progression-free survival time was 6.5 months (95% CI 2.6–10.4). In the IFN/FU group, the median progression-free survival

Table 3. Factorial analysis of predictors of response

	Response rate %	Univariate analysis, p value[a]	Multivariate analysis, p value[b]	Odds ratio (95% CI)
IFN/FU + CDDP/IFN/FU	45.6/25.6	0.0302	0.0268	2.5 (1.1–6.0)
Gender (male/female)	35.8/31.6	0.7979		
Age (<65/≥65 years)	31.1/39.6	0.4318		
Primary/recurrence	32.6/36.6	0.6909		
Prior TACE (+/–)	35.4/36.7	1.00		
Prior chemotherapy (+/–)	57.1/33.6	0.2382		
HCV-Ab (positive/negative)	40.3/27.7	0.2315		
HBsAg (positive/negative)	38.2/33.7	0.6722		
Liver cirrhosis (+/–)	37.6/23.8	0.3134		
Child-Pugh class (A/B, C)	41.5/26.5	0.115		
LCSGJ TNM stage (II, III/IVA, IVB)	46.7/22.2	0.0102	0.2877	1.3 (0.4–4.0)
Diameter of tumor (<50/≥50 mm)	44.0/17.9	0.008	0.1817	2.2 (0.7–7.0)
Major portal vein invasion (+/–)	16.1/42.2	0.0143	0.1266	1.8 (0.5–6.8)
Lymph node metastasis (+/–)	33.3/35.2	1.00		
Distant metastasis (+/–)	16.7/37.3	0.2098		
Albumin (<3.5/≥3.5 g/dl)	27.6/42.9	0.1165		
Total bilirubin (<1.5/≥1.5 mg/dl)	39.2/25.7	0.2038		
Active prothrombin (<70/≥70%)	26.8/39.7	0.2203		
Platelets (<10/≥10 × 10^4/μl)	33.3/36.7	0.8444		
AST (<80/≥80 IU/l)	40.8/25.6	0.1096		
ALT (<80/≥80 IU/l)	33.7/40.0	0.6372		
DCP (<100/≥100 mAU/ml)	42.5/57.5	0.5511		
AFP (<400/≥400 ng/ml)	38.7/30.8	0.4334		
AFP-L3 (<30/≥30%)	46.6/22.4	0.0094	0.2898	1.7 (0.7–4.4)

HBsAg = Hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein. [a] Fisher's exact test. [b] Logistic procedure model.

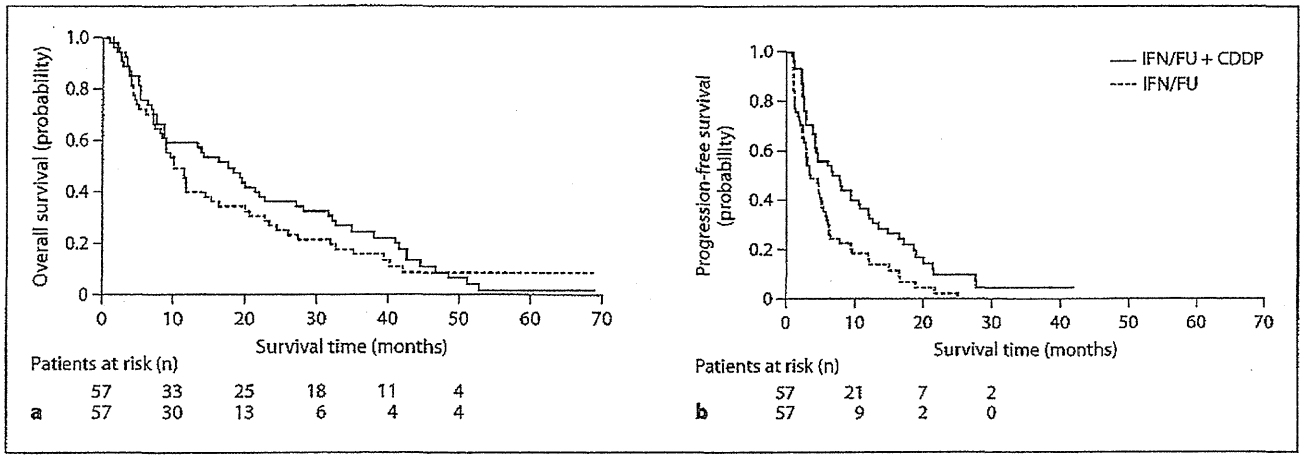

Fig. 3. Kaplan-Meier analysis of overall survival (**a**) and progression-free survival (**b**) according to the different chemotherapeutic regimens.

Table 4. Most common adverse events

Adverse event	IFN/FU + CDDP (n = 57)		IFN/FU (n = 57)	
	any grade	CTC grade 3–4	any grade	CTC grade 3–4
Neutropenia	44 (77.2)	17 (29.8)	37 (64.9)	19 (33.3)
Leukopenia	43 (75.4)	12 (21.1)	38 (66.7)	18 (31.6)
Reduced hemoglobin	52 (91.2)[a]	4 (7.0)	43 (75.4)[a]	2 (3.5)
Thrombocytopenia	50 (89.5)	26 (45.6)[b]	48 (84.2)	13 (22.8)[b]
Prothrombin time	30 (52.6)	3 (5.3)	32 (56.1)	1 (1.8)
Asthenia	34 (59.6)[a]	1 (1.8)	21 (36.8)[a]	3 (5.3)
Fever	41 (71.9)	1 (1.8)	37 (64.9)	0
Nausea	32 (56.1)[a]	10 (17.5)	22 (38.6)[a]	3 (5.3)
Vomiting	15 (26.3)[a]	4 (7.0)	4 (7.0)[a]	1 (1.8)
Mucositis	22 (38.6)[a]	3 (5.3)	9 (15.8)[a]	1 (1.8)
Liver function	42 (73.7)	4 (7.0)	43 (75.4)	10 (17.5)
Creatinine elevation	10 (17.5)[a]	0	2 (3.5)[a]	0
Peptic ulcer	6 (10.5)	0	1 (1.8)	0

Values represent numbers of events with percentages in parentheses. [a] $p < 0.05$ (Wilcoxon rank sum test); [b] $p < 0.05$ (Fisher's exact test). CTC = Common Toxicity Criteria.

time was 3.3 months (95% CI −0.6 to 7.2). The progression-free survival period was significantly longer in the IFN/FU + CDDP group than in the IFN/FU group ($p = 0.0048$, log-rank test; hazard ratio 0.57, 95% CI 0.38–0.85; fig. 3b).

As predictors for survival, a multivariate analysis showed that positivity for hepatitis C virus antibody (HCV-Ab), an albumin level of 3.5 g/dl or more and an aspartate aminotransferase (AST) value of lower than 80 IU/l were associated with improved survival (table 5).

Discussion

The present study showed that the addition of CDDP to IFN combined with HAIC using FU significantly enhanced the antitumor effect from 24.6 to 45.6%. The response rates obtained in previous studies of HAIC involving at least 30 patients varied from 14 to 71% [8–17]. Regarding the use of IFN combined with HAIC using FU, Obi et al. [13] used this treatment in patients with advanced HCC and a tumor embolus in the main trunk or the first branch of the portal vein and achieved a response rate of 52.6%. Ota et al. [18] also used IFN combined with HAIC using FU for similar cases of advanced HCC and reported a response rate of 43.6%. We previously reported a response rate of 45% in 34 patients who underwent multidrug HAIC using FU and CDDP in combination with IFN treatment [11]. Uka et al. [21] used IFN in combination with HAIC using FU in 55 patients who had a tumor embolus of the portal vein and reported a response rate of 29%. The response rates obtained in the present study were similar to those obtained in the report by Uka et al. [21] and lower than those obtained in the other two reports. This discrepancy may be explained by the different criteria used to evaluate antitumor efficacy, as Uka et al. [21] suggested in their discussion. Obi et al. [13] and Ota et al. [18] used the Eastern Clinical Oncology Group criteria, whereas Uka et al. [21] and the present study used the Response Evaluation Criteria in Solid Tumors.

The combined use of FU and IFN is reportedly beneficial because IFN serves as a modulator to enhance the antitumor effect of FU. More specifically, IFN induces p53, which enhances apoptosis by FU, and influences the cell cycle via $p27^{Kip1}$ or apoptosis via Bcl-xL [22, 23]. From a clinical perspective, Takaki-Hamade et al. [24] and Eun et al. [25] concluded that combined IFN treatment did not have an incremental effect. Thus, the benefit of adding IFN to HAIC with FU has not been proven clinically. However, experimental data suggest that IFN should enhance the antitumor effect of FU [22, 26], and this supports the current use of IFN-combined HAIC in clinical practice.

On the other hand, with regard to the effect of CDDP combined with FU in a clinical setting, Ando et al. [8] used HAIC with FU combined with low-dose CDDP for the treatment of patients with advanced HCC and a portal

Table 5. Factorial analysis of predictors of survival

	MST, months	Univariate analysis, p value[a]	Multivariate analysis, p value[b]	Hazard ratio (95% CI)
IFN/FU + CDDP/IFN/FU	17.6/10.5	0.522		
Gender (male/female)	12.0/12.0	0.236		
Age (<65/≥65 years)	9.9/19.5	0.115		
Primary/recurrence	7.7/16.5	0.394		
Prior TACE (+/–)	14.4/12.0	0.491		
Prior chemotherapy (+/–)	18.6/12.0	0.936		
HCV-Ab (positive/negative)	19.5/7.6	0.0049	0.0219	0.60 (0.39–0.93)
HBsAg (positive/negative)	7.6/15.4	0.1145		
Liver cirrhosis (+/–)	13.7/9.0	0.5063		
Child-Pugh class (A/B, C)	18.6/9.2	0.0636		
LCSGJ TNM stage (II, III/IVA, IVB)	19.4/7.5	0.0019	0.6326	0.87 (0.49–1.54)
Diameter of tumor (<50/≥50 mm)	19.4/5.8	0.0014	0.1068	0.64 (0.37–1.10)
Major portal vein invasion (+/–)	5.1/18.6	0.0005	0.3203	0.73 (0.40–1.35)
Lymph node metastasis (+/–)	4.5/12.0	0.0789		
Distant metastasis (+/–)	4.5/14.0	0.0037	0.1806	0.60 (0.29–1.27)
Albumin (<3.5/≥3.5 g/dl)	9.3/16.5	0.0200	0.0017	0.50 (0.32–0.77)
Total bilirubin (<1.5/≥1.5 mg/dl)	15.4/9.5	0.2774		
Active prothrombin (<70/≥70%)	9.3/14.5	0.9470		
Platelets (<10/≥10 × 10^4/μl)	16.5/10.5	0.6273		
AST (<80/≥80 IU/l)	19.4/7.4	0.0056	0.0356	0.62 (0.39–0.97)
ALT (<80/≥80 IU/l)	13.7/9.5	0.8973		
DCP (<100/≥100 mAU/ml)	20.0/9.4	0.2294		
AFP (<400/≥400 ng/ml)	21.5/6.6	0.0002	0.1588	0.69 (0.41–1.16)
AFP-L3 (<30/≥30%)	20.8/7.5	0.0002	0.0730	0.61 (0.35–1.05)

HBsAg = Hepatitis B surface antigen; LCSGJ = Liver Cancer Study Group of Japan; Major portal vein invasion = tumor invasion in main trunk or 1st branches of portal vein; ALT = alanine aminotransferase; DCP = des-gamma-carboxy prothrombin; AFP = α-fetoprotein. [a] Log-rank test. [b] Cox proportional hazard model.

tumor embolus and reported a response rate of 48%. Following their report, several other reports on HAIC with FU combined with low-dose CDDP were published, with reported response rates ranging from 38.5 to 71% [14, 16, 17, 27]. Experimental studies have shown that low-dose CDDP blocks methionine transport into the cell, causing a decrease in intracellular methionine and an increase in reduced folic acid, thus serving as a modulator of FU to enhance its antitumor efficacy [28]. It has also been reported that low-dose CDDP is involved in the inhibition of p53-mediated apoptosis and drug resistance [29]. The present study used two agents, IFN and CDDP, in combination with FU. Although IFN and CDDP seem to enhance the antitumor effect of FU through these pathways, a large amount of basic experimental research on FU combined with these two agents remains to be performed.

Our present study showed that the antitumor effect was significantly greater and the progression-free survival time was significantly longer in the IFN/FU + CDDP group. However, there was no statistically significant difference in the overall survival time. Subgroup analysis also did not show survival benefit in the IFN + CDDP group. Since there were no limitations on treatment after the end of the protocol treatment, 88 of the 114 patients (77.2%) underwent some treatment subsequently, and 34 patients (59.6%) in the IFN/FU group received HAIC (mainly IFN/FU + CDDP) eventually. This might have had some effects on the results for overall survival.

The factors that improved survival in this study included positivity for HCV-Ab, an albumin level of 3.5 g/dl or more and an AST value of lower than 80 IU/l. Previous reports have documented the presence of response to chemotherapy, the Cancer of the Liver Italian Program score, the Okuda stage, the Child-Pugh score and α-fetoprotein as prognostic factors for HAIC in advanced HCC [30, 31]. Obi et al. [13] also reported that positivity for HCV-Ab was a predictor of a CR to IFN combined with HAIC using FU. Uka et al. [21] reported that positivity

for HCV-Ab was a factor associated with the early antitumor effect, progression-free time and overall survival after IFN combined with HAIC. Thus, positivity for HCV-Ab was determined to be a factor that improved prognosis. A possible explanation for this discrepancy may be that viral differences between hepatitis B virus and HCV may be involved in the heterogeneity of the anticancer drug sensitivity of HCC, or differences in the cytokine patterns of hepatitis B virus and HCV infections may influence the effect of IFN [32–35]. However, the true explanation remains unclear. In connection with an AST value of lower than 80 IU/l, Cheong et al. [36] also reported that low levels of AST and alkaline phosphatase were associated with long-term survival exceeding 8 months in a study examining chemotherapy including HAIC for the treatment of patients with advanced HCC. The basis of their findings requires further investigation.

Most patients with HCC have concomitant hepatic cirrhosis and thus have pancytopenia. Therefore, with regard to adverse events, we expected to see enhanced blood toxicity when IFN and CDDP were added to FU. Thus, this study showed a significantly higher frequency of cytopenia in the IFN/FU + CDDP group. However, as far as severe hematologic toxicities of grade 3 or 4 were concerned, thrombocytopenia alone was significantly more frequent in the IFN/FU + CDDP group, but no complications secondary to thrombocytopenia occurred. Although some nonhematologic toxicities were significantly more frequent in the IFN/FU + CDDP group, these adverse events were controllable. Thus, IFN combined with HAIC using FU and CDDP seems to be tolerable with regard to the occurrence of adverse events. The frequency of grade 3 or 4 toxicity with IFN-combined HAIC in our study was higher than that with sorafenib therapy reported previously [6, 7]. We enrolled 45 patients (39.5%) in Child-Pugh class B, and the pretreatment blood cell count in patients of Child-Pugh class B was generally lower than that of those patients in Child-Pugh class A. In addition, IFN has the effect of decreasing the blood cell count, especially neutrophils and platelets. However, these toxicities were controllable and there were no toxicity-related deaths.

In conclusion, the results of this phase II randomized clinical study on the effect of adding CDDP to IFN in combination with HAIC using FU for the treatment of advanced HCC show that the addition of CDDP significantly increases the antitumor effect of the treatment and results in a significant improvement in the progression-free survival time. Although there was no significant difference in the overall survival time of the two treatment groups, the survival benefit of IFN combined with HAIC using CDDP should be examined in comparison with systemic therapy using sorafenib, the current standard treatment for advanced HCC. In this regard, a multicenter study of hepatic arterial infusion of FU versus sorafenib therapy is now under way in Japan, and the results are awaited.

Disclosure Statement

There are no financial disclosures from any authors. There are no conflicts of interest for any authors.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.

2 Bosch FX, Ribes J, Diaz M, Cleries R: Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127:S5–S16.

3 Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB: Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. Gastroenterology 2004;127:1372–1380.

4 Arii S, Sata M, Sakamoto M, Shimada M, Kumada T, Shiina S, Yamashita T, Kokudo N, Tanaka M, Takayama T, Kudo M: Management of hepatocellular carcinoma: Report of Consensus Meeting in the 45th Annual Meeting of the Japan Society of Hepatology (2009). Hepatol Res 2010;40:667–685.

5 Ikai I, Arii S, Okazaki M, Okita K, Omata M, Kojiro M, Takayasu K, Nakanuma Y, Makuuchi M, Matsuyama Y, Monden M, Kudo M: Report of the 17th Nationwide Follow-up Survey of Primary Liver Cancer in Japan. Hepatol Res 2007;37:676–691.

6 Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25–34.

7 Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–390.

8 Ando E, Tanaka M, Yamashita F, Kuromatsu R, Yutani S, Fukumori K, Sumie S, Yano Y, Okuda K, Sata M: Hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma with portal vein tumor thrombosis: analysis of 48 cases. Cancer 2002;95:588–595.

9 Chung YH, Song IH, Song BC, Lee GC, Koh MS, Yoon HK, Lee YS, Sung KB, Suh DJ: Combined therapy consisting of intraarterial cisplatin infusion and systemic interferon-alpha for hepatocellular carcinoma patients with major portal vein thrombosis or distant metastasis. Cancer 2000;88:1986–1991.

10 Court WS, Order SE, Siegel JA, Johnson E, DeNittis AS, Principato R, Martz K, Zeiger LS: Remission and survival following monthly intraarterial cisplatinum in nonresectable hepatoma. Cancer Invest 2002;20:613–625.

11 Kaneko S, Urabe T, Kobayashi K: Combination chemotherapy for advanced hepatocellular carcinoma complicated by major portal vein thrombosis. Oncology 2002;62(suppl 1):69–73.

12 Lin CP, Yu HC, Cheng JS, Lai KH, Lo GH, Hsu PI, Lin CK, Chen HH, Lo CC, Liang HL, Tseng HH: Clinical effects of intra-arterial infusion chemotherapy with cisplatin, mitomycin C, leucovorin and 5-flourouracil for unresectable advanced hepatocellular carcinoma. J Chin Med Assoc 2004;67:602–610.

13 Obi S, Yoshida H, Toune R, Unuma T, Kanda M, Sato S, Tateishi R, Teratani T, Shiina S, Omata M: Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. Cancer 2006;106:1990–1997.

14 Okuda K, Tanaka M, Shibata J, Ando E, Ogata T, Kinoshita H, Eriguchi N, Aoyagi S, Tanikawa K: Hepatic arterial infusion chemotherapy with continuous low dose administration of cisplatin and 5-fluorouracil for multiple recurrence of hepatocellular carcinoma after surgical treatment. Oncol Rep 1999;6:587–591.

15 Park JY, Ahn SH, Yoon YJ, Kim JK, Lee HW, Lee do Y, Chon CY, Moon YM, Han KH: Repetitive short-course hepatic arterial infusion chemotherapy with high-dose 5-fluorouracil and cisplatin in patients with advanced hepatocellular carcinoma. Cancer 2007;110:129–137.

16 Sumie S, Yamashita F, Ando E, Tanaka M, Yano Y, Fukumori K, Sata M: Interventional radiology for advanced hepatocellular carcinoma: comparison of hepatic artery infusion chemotherapy and transcatheter arterial lipiodol chemoembolization. AJR Am J Roentgenol 2003;181:1327–1334.

17 Tanioka H, Tsuji A, Morita S, Horimi T, Takamatsu M, Shirasaka T, Mizushima T, Ochi K, Kiura K, Tanimoto M: Combination chemotherapy with continuous 5-fluorouracil and low-dose cisplatin infusion for advanced hepatocellular carcinoma. Anticancer Res 2003;23:1891–1897.

18 Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, Nakamura M, Damdinsuren B, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Wakasa K, Monden M: Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type I interferon receptor expression. Br J Cancer 2005;93:557–564.

19 Tzoracoleftherakis EE, Spiliotis JD, Kyriakopoulou T, Kakkos SK: Intra-arterial versus systemic chemotherapy for non-operable hepatocellular carcinoma. Hepatogastroenterology 1999;46:1122–1125.

20 Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG: New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–216.

21 Uka K, Aikata H, Takaki S, Kawaoka T, Saneto H, Miki D, Takahashi S, Toyota N, Ito K, Chayama K: Systemic gemcitabine combined with intra-arterial low-dose cisplatin and 5-fluorouracil for advanced hepatocellular carcinoma: seven cases. World J Gastroenterol 2008;14:2602–2608.

22 Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K, Taniguchi T: Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. Nature 2003;424:516–523.

23 Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M, Monden M: Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with upregulation of p27Kip1 in human hepatocellular carcinoma cells. Clin Cancer Res 2000;6:2881–2890.

24 Takaki-Hamabe S, Yamasaki T, Saeki I, Harima Y, Okita K, Terai S, Sakaida I: Hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma: is the addition of subcutaneous interferon-alpha-2b beneficial? Hepatol Res 2009;39:223–230.

25 Eun JR, Lee HJ, Moon HJ, Kim TN, Kim JW, Chang JC: Hepatic arterial infusion chemotherapy using high-dose 5-fluorouracil and cisplatin with or without interferon-alpha for the treatment of advanced hepatocellular carcinoma with portal vein tumor thrombosis. Scand J Gastroenterol 2009;44:1477–1486.

26 Moriyama M, Hoshida Y, Kato N, Otsuka M, Yoshida H, Kawabe T, Omata M: Genes associated with human hepatocellular carcinoma cell chemosensitivity to 5-fluorouracil plus interferon-alpha combination chemotherapy. Int J Oncol 2004;25:1279–1287.

27 Ueshima K, Kudo M, Takita M, Nagai T, Tatsumi C, Ueda T, Kitai S, Ishikawa E, Yada N, Inoue T, Hagiwara S, Minami Y, Chung H: Hepatic arterial infusion chemotherapy using low-dose 5-fluorouracil and cisplatin for advanced hepatocellular carcinoma. Oncology 2010;78(suppl 1):148–153.

28 Nishiyama M, Yamamoto W, Park JS, Okamoto R, Hanaoka H, Takano H, Saito N, Matsukawa M, Shirasaka T, Kurihara M: Low-dose cisplatin and 5-fluorouracil in combination can repress increased gene expression of cellular resistance determinants to themselves. Clin Cancer Res 1999;5:2620–2628.

29 Matsuhashi N, Saio M, Matsuo A, Sugiyama Y, Saji S: Expression of p53 protein as a predictor of the response to 5-fluorouracil and cisplatin chemotherapy in human gastrointestinal cancer cell lines evaluated with apoptosis by use of thin layer collagen gel. Int J Oncol 2004;24:807–813.

30 Hamada A, Yamakado K, Nakatsuka A, Takaki H, Akeboshi M, Takeda K: Hepatic arterial infusion chemotherapy with use of an implanted port system in patients with advanced hepatocellular carcinoma: prognostic factors. J Vasc Interv Radiol 2004;15:835–841.

31 Yamasaki T, Kimura T, Kurokawa F, Aoyama K, Ishikawa T, Tajima K, Yokoyama Y, Takami T, Omori K, Kawaguchi K, Tsuchiya M, Terai S, Sakaida I, Okita K: Prognostic factors in patients with advanced hepatocellular carcinoma receiving hepatic arterial infusion chemotherapy. J Gastroenterol 2005;40:70–78.

32 Buendia MA: Hepatitis B viruses and cancerogenesis. Biomed Pharmacother 1998;52:34–43.

33 Falasca K, Ucciferri C, Dalessandro M, Zingariello P, Mancino P, Petrarca C, Pizzigallo E, Conti P, Vecchiet J: Cytokine patterns correlate with liver damage in patients with chronic hepatitis B and C. Ann Clin Lab Sci 2006;36:144–150.

34 Robinson WS: Molecular events in the pathogenesis of hepadnavirus-associated hepatocellular carcinoma. Annu Rev Med 1994;45:297–323.

35 Someya T, Ikeda K, Saitoh S, Kobayashi M, Hosaka T, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Arase Y, Kumada H: Interferon lowers tumor recurrence rate after surgical resection or ablation of hepatocellular carcinoma: a pilot study of patients with hepatitis B virus-related cirrhosis. J Gastroenterol 2006;41:1206–1213.

36 Cheong JY, Lee KM, Cho SW, Won JH, Kim JK, Wang HJ, Hahm KB, Kim JH: Survival benefits of intra-arterial infusion chemotherapy in patients with advanced hepatocellular carcinoma with portal vein tumor thrombosis. Hepatol Res 2005;32:127–133.

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Enhancement of tumor-specific T-cell responses by transcatheter arterial embolization with dendritic cell infusion for hepatocellular carcinoma

Eishiro Mizukoshi[1], Yasunari Nakamoto[1], Kuniaki Arai[1], Tatsuya Yamashita[1], Naofumi Mukaida[2], Kouji Matsushima[3], Osamu Matsui[4] and Shuichi Kaneko[1]

[1] Department of Disease Control and Homeostasis, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan
[2] Division of Molecular Bioregulation, Cancer Research Institute, Kanazawa University, Kanazawa, Japan
[3] Department of Molecular Preventive Medicine, Graduate School of Medicine, Tokyo University, Tokyo, Japan
[4] Department of Radiology, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan

Transcatheter arterial embolization (TAE) destroys a tumor by the induction of necrosis and/or apoptosis and causes inflammation with cytokine production, which may favor immune activation and presentation of tumor-specific antigens. In the current study, we attempted to identify the effect of TAE on tumor-specific T-cell responses and the additional effect of dendritic cell (DC) infusion performed during TAE. The prevalence of tumor antigen-specific T cells was determined by interferon-γ enzyme-linked immunospot analysis using alpha-fetoprotein (AFP) and tumor antigen-derived peptides in 20 and 13 patients with hepatocellular carcinoma (HCC) who received TAE and TAE with DC infusion, respectively. The increased frequency of AFP-specific T cells was observed in 6 of 20 patients after TAE. It was observed more frequently in patients with DC infusion than in those with TAE alone. However, tumor recurrence was not completely prevented in patients albeit displayed enhanced immune responses. The evidence that the enhanced immune responses were transient and attenuated within 3 months was provided in time-course analysis. In conclusion, TAE with DC infusion enhances the tumor-specific immune responses more effectively than TAE alone. Although the effect is not sufficient to prevent HCC recurrence, these results may contribute to the development of novel immunotherapeutic approach for HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignancies and has gained major clinical interest because of its increasing incidence. Although current advances in therapeutic modalities have improved the prognosis of patients with HCC, the survival rate is still unsatisfactory.[1–4] One of the reasons for the poor prognosis is the high rate of recurrence after treatment.[5] Therefore, the development of new antitumor therapies to protect against recurrence is important to improve the prognosis for HCC.

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Several recent studies of cancer treatment causing tumor necrosis or apoptosis have shown that they induce the activation of tumor-specific immune responses.[6–10] The mechanism to activate host immune responses against tumors is still unknown; however, several studies *in vitro* or *in vivo* suggest that cytokine production, attracting leukocyte infiltration, increase of tumor antigen uptake by macrophages or dendritic cells (DCs) and release of heat shock protein caused by inflammation at the tumor site are associated with the phenomenon.[11–17]

Transcatheter arterial embolization (TAE) has been used extensively in the Western world and Asia to treat unresectable HCCs.[18–20] Although several previous randomized controlled trials have failed to show a survival benefit in patients treated with TAE compared to untreated patients,[21,22] recent studies demonstrated a survival benefit for TAE *versus* conservative treatment in carefully selected patients.[23–25]

Histological assessment of resected HCC after TAE shows that the treatment induces necrotic and apoptotic changes in the tumor.[26–29] Moreover, it is reported that the serum levels of macrophage-colony stimulating factor and the lipopolysaccharide-stimulated production of interleukin-1 beta, IL-6 and tumor necrosis factor-alpha in peripheral whole blood were increased after TAE.[30–32] Taken together with the previously described knowledge of immune responses after treatment to induce tumor necrosis or apoptosis, these observations support the hypothesis that the induction of apoptotic or necrotic cell death and inflammatory cytokines by TAE favors immune activation and induction of tumor-specific T-cell

Key words: immune response, AFP, CTL, immunotherapy, epitope
Abbreviations: HLA: human leukocyte antigens; IFN: interferon; HCV: hepatitis C virus; ELISPOT: enzyme-linked immunospot; TAE: transcatheter arterial embolization; MRP: multidrug resistance-associated protein; hTERT: human telomerase reverse transcriptase
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Correspondence to: Shuichi Kaneko, Department of Disease Control and Homeostasis, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan,
Fax: +81-76-2344250, E-mail: skaneko@m-kanazawa.jp

responses. In a previous study, we also made a preliminary report that immune responses specific for tumor antigens were enhanced after HCC treatments.[7,10] In addition, we have recently developed a new immunotherapeutic approach for HCC using DC infusion performed during TAE, showing the potential to enhance tumor-specific immune responses.[7]

In the current study, we first attempted to identify the effect of TAE for tumor-specific T-cell responses in patients with HCC. Next, we examined the additional effects of DC infusion to the tumor site after TAE. Finally, we analyzed the relationship between clinical characteristics of patients and T-cell responses after TAE and evaluated whether the activation of tumor-specific T-cell responses can prevent HCC recurrence.

Material and Methods

Patient population

The study examined 33 patients with HCC, consisting of 25 men and 8 women ranging from 48 to 83 years old with a mean age of 66 ± 9 years. Twenty patients were treated by TAE. Thirteen patients were treated by TAE with DC infusion as a part of clinical study, which was approved by ethical committee of Kanazawa University Graduate School of Medical Science and registered in September 2003. The patients who received TAE with DC infusion were selected according to the criteria we previously reported.[7] All subjects were negative for Abs to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration.

Treatment of hepatocellular carcinoma

HCCs were detected by imaging modalities such as dynamic CT scan, MR imaging and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens, surgical resection or autopsy in 18 cases. For the remaining 15 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[33] The tumor size was categorized as "small" (≤2 cm) or "large" (>2 cm), and tumor multiplicity was categorized as "multiple" (≥2 nodules) or "solitary" (single nodule). The TNM stage was classified according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version).[34]

Twenty patients were treated by TAE as previously described.[19,35] In brief, after evaluation of the feeding arteries and surrounding vascular anatomy, a microcatheter (Microferret, Cook, Bloomington, IN) was inserted into the segmental or subsegmental artery with a coaxial method using a 0.016-inch guidewire (Radifocus GT wire, Terumo, Tokyo, Japan). A mixture of the anticancer drug and iodized oil was administered, and the feeding artery was embolized with gelatin sponge particles (Gelfoam; Pharmacia Upjohn, Kalamazoo, MI). The mixture of anticancer drug and iodized oil contained 10–30 mg of Epirubicin (Farmorubicin; Kyowa Hakko Kogyo, Tokyo, Japan), 1–3 ml of iodized oil (Lipiodol Ultra Fluide) and 0.5–1.0 ml of iohexol (Omnipaque 300).

Preparation and injection of autologous DCs

DCs were generated as previously described.[7] In 6 patients, DCs were pulsed with 0.1 KE/ml OK-432 (Chugai Pharmaceutical, Tokyo, Japan), which is a biological response modifier derived from the weakly virulent Su strain of Streptococcus pyogenes,[36,37] for 3 days before injection. The cells were harvested for injection; 5×10^6 cells were reconstituted in 5-ml normal saline containing 1% autologous plasma, mixed with gelatin sponge particles and infused through an arterial catheter following iodized oil injection during TAE.

After TAE or TAE with DC infusion, 26 patients received percutaneous tumor ablation by ethanol injection (PEIT), microwave coagulation (MCT) or radiofrequency (RF). Twenty-one patients were diagnosed with complete necrosis of the tumor lesion using dynamic CT after the completion of treatment. Follow-ups were conducted at outpatient clinics using blood tests and dynamic CT every 3 months for 1 year.

Laboratory and virologic testing

Blood samples were tested for HBsAg and HCVAb by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of PBMC from patients was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda. The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.[38] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.*[39]

Interferon-γ enzyme-linked immunospot assay

The prevalence of tumor antigen-specific T cells was determined by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis (Mabtech, Nacka, Sweden) as previously described.[10,40] HLA-A24-restricted AFP-derived peptides (10 μg/ml), which were AFP_{357} (EYSRRHPQL), AFP_{403} (KYIQESQAL) and AFP_{434} (AYTKKAPQL),[10] and 20 μg/ml AFP derived from human placenta (Morinaga Institute of Biological Science, Yokohama, Japan, purity >98%) were added directly to the wells. These 3 AFP-derived peptides could induce CTLs showing cytotoxicity against hepatoma cells and were frequently recognized by PBMCs of patients with HCC as we previously reported,[10] and therefore, we selected them as an immunogenic peptide. The HLA-A24-restricted AFP and CMV-derived peptides were used only for HLA-A24 or A23 positive patients. Other tumor antigen-derived peptides consisted of $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), $MRP3_{765}$ (VYSDADIFL), $hTERT_{167}$ (AYQVCGPPL), $hTERT_{324}$

(VYAETKHFL) and $hTERT_{461}$ (VYGFVRACL), which we previously reported that they were useful for analyzing host immune responses to HCC.[40,41]

PBMCs were added to the wells at 3×10^5 cells/well. In the assay using PBMC depleted $CD4^+$ or $CD8^+$ cells, the number of cells was adjusted to 3×10^5 cells/well after the depletion. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system using CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. After the depletion, 1×10^6 cells were stained with CD4 and CD8 antibodies (Becton Dickinson, Tokyo, Japan) and analyzed by FACSCalibur (Becton Dickinson, Tokyo, Japan) to confirm the ratio of $CD4^+$ and $CD8^+$ cells. Data analysis was undertaken with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Plates were analyzed with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least 2-fold greater than the number of spots in the absence of antigen. Negative controls consisted of incubation of PBMCs with a peptide representing an HLA-A24-restricted epitope derived from HIV envelope protein ($HIVenv_{584}$) and were always <5 spots per 3×10^5 cells.[42] The positive controls consisted of 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$).[43] All peptides used in this study were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). ELISPOT analysis was performed before and 2–4 weeks after TAE. In patients receiving additional treatment for complete ablation of tumor, analysis was performed just before the additional treatment. An increase of antigen-specific T cells was defined as significant when T-cell responses changed to positive or if the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment.

Statistical analysis

Unpaired Student's *t*-test was used to analyze the effect of variables on immune responses in patients with HCC. Fisher's exact test (2-sided *p*-value) was used to analyze the frequency of positive immune responses in patients between with TAE and TAE with DC infusion.

Results

T-cell responses to AFP in the patients who received TAE

The frequency of AFP-specific T cells before and after TAE was tested *ex vivo* in an IFN-γ ELISPOT assay. The serum AFP level and number of peripheral lymphocytes and antigen-specific T cells are shown in Table 1. Before treatment, 2 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein in 20 patients (Patients 1–20). After treatment, a T-cell response to AFP-derived peptides and protein was detected in 4 and 3 patients, respectively.

When an increase of antigen-specific T cells was defined as significant if T-cell responses changed to positive or the number of spots detected after TAE was at least 2-fold greater than the number of spots detected before treatment, 6 of 20 (30%) patients (Patients 4, 6, 7, 11, 18 and 20) showed a significant increasing of AFP-specific T-cell frequency after treatment. It was observed even in the patient (Patients 6, 7 and 18) who had no T cells specific to corresponding AFP-derived peptides before treatment. When a decrease of antigen-specific T cells was defined as significant if T-cell responses changed from positive to negative or the number of spots detected after TAE was less than half of the number of spots detected before treatment, 4 of 20 (20%) patients (Patients 5, 14, 15 and 16) showed a significant decreasing of AFP-specific T-cell frequency after treatment.

AFP-specific IFN-γ-producing T cells were also analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system, and the results were confirmed by flow cytometric analysis (Fig. 1*a*). After depletion of $CD4^+$ or $CD8^+$ cells, the ratio of each cell population was decreased to less than 0.1% of PBMCs. The IFN-γ ELISPOT assay showed that IFN-γ-producing T cells against AFP consisted of both $CD8^+$ and $CD4^+$ cells (Fig. 1*b*).

To confirm the effect of TAE for host immune responses to HCC, we also examined the frequency of tumor antigen-specific T cells in 4 patients (Patients 5, 8, 10 and 14) using MRP3- or hTERT-derived peptides that we previously identified as useful for analyzing host immune responses to HCC.[40,41] A significant increasing of MRP3- or hTERT-specific T-cell frequency was observed in all patients after TAE (Table 2).

T-cell responses to AFP in the patients who received TAE with DC infusion

In 13 patients receiving TAE with DC infusion (Patients 21–33), 2 patients showed a specific T-cell response with AFP-derived peptides and 2 patients with protein before treatment (Table 3). After treatment, 8 patients showed a specific T-cell response to AFP-derived peptides and 3 patients to protein.

Next, we compared TAE with DC infusion with TAE alone regarding the effect to AFP-specific immune response. Table 4 shows the clinical features of patients with HCC who received TAE and TAE with DC infusion and they were not statistically different except liver function.

The frequency of patients who showed both positive and increasing T-cell response with AFP-derived peptides or protein after treatment was significantly higher in patients receiving TAE with DC infusion than in those receiving TAE alone ($p = 0.04$) (Fig. 2*a*). On the other hand, the frequency of patients who showed both positive and increasing T-cell

Table 1. T cell response to AFP and AFP-derived peptides by ELISPOT assay before and after TAE

				Before treatment								After treatment							
Patient	HLA	Additional treatment	Complete ablation	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT	AFP (ng/ml)	Lymph. (μl^{-1})	AFP_{357}	AFP_{403}	AFP_{434}	AFP	$CMVpp65_{328}$	TT
1	A2	RF	C	<10	1,600	ND	ND	ND	1	ND	0	<10	1,400	ND	ND	ND	0	ND	1
2	A26,A31	RF	C	61	1,700	ND	ND	ND	0	ND	**13**	23	900	ND	ND	ND	0	ND	0
3	A11,A26	No	–	100	1,700	ND	ND	ND	5	ND	1	50	1,500	ND	ND	ND	0	ND	0
4	A24	RF	C	18	700	0	7	0	6	0	**25**	16	500	1	**10**	1	1	2	**16**
5	A24,A33	RF	C	2,357	1,200	**13**	2	6	0	**13**	0	700	1,100	2	1	1	0	9	0
6	A24	RF	C	14	1,800	0	0	0	0	0	**42**	<10	1,400	**53**	**27**	**38**	**14**	**36**	**108**
7	A23,A33	No	–	96	500	0	0	0	5	**291**	0	138	800	**46**	0	0	3	**484**	0
8	A24,A26	No	–	142	600	1	0	0	0	0	0	126	500	2	0	0	0	**166**	1
9	A2,A24	RF	C	<10	700	6	1	0	0	9	0	<10	700	0	0	0	0	**32**	**15**
10	A24	PEIT	C	<10	1,300	8	4	8	8	**146**	5	<10	1,300	0	1	1	0	1	1
11	A24,A26	PEIT	N	18	1,100	0	0	0	1	ND	0	13	400	0	0	0	**15**	**10**	**55**
12	A24,A33	RF	N	11	800	3	2	0	4	**94**	**10**	11	700	0	0	0	0	**24**	0
13	A11,A24	PEIT	C	52	1,300	0	2	5	1	2	0	24	1,200	0	0	0	0	0	3
14	A24	RF	C	54	2,400	**25**	5	4	8	**12**	0	67	1,700	0	0	0	0	0	0
15	A2,A24	RF	N	62	1,200	0	3	0	**25**	2	3	14	800	0	0	0	8	0	0
16	A3,A24	RF	C	2,876	900	0	1	0	**13**	0	5	3,285	700	0	0	0	0	0	0
17	A24,A33	No	–	205	400	4	2	3	2	**26**	9	220	100	2	1	0	1	**39**	1
18	A24,A30	RF	C	18	1,100	4	0	3	8	**14**	7	13	900	1	**16**	1	5	**12**	0
19	A2,A24	RF	C	330	1,500	2	0	0	0	**18**	1	36	1,100	0	4	0	3	8	1
20	A2,A33	RF	C	10	1,400	ND	ND	ND	**10**	ND	**68**	<10	800	ND	ND	ND	**31**	ND	**101**

Abbreviations: Lymph., number of lymphocytes; RF, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy; No, no treatment; C, completed; N, not completed; –, not determined; ND, not done. The bold letters show the positive responses in ELISPOT assays.

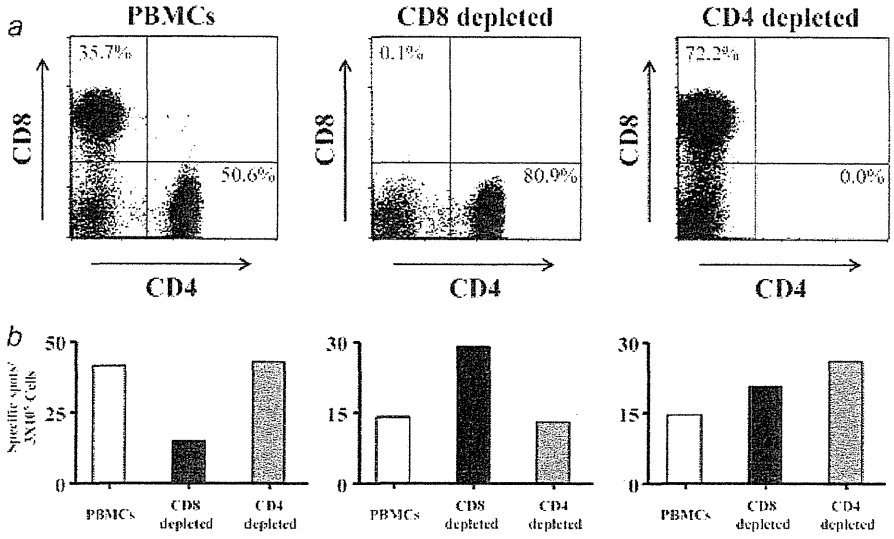

Figure 1. IFN-γ production of CD4- or CD8-depleted T cells against whole AFP. AFP-specific IFN-γ-producing T cells were analyzed by ELISPOT assay using PBMC depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cells is responsive to whole AFP. Depletion of $CD4^+$ or $CD8^+$ cells was performed by MACS separation system and the results were confirmed by flow cytometric analysis (*a*). IFN-γ ELISPOT assay using nontreated PBMCs and PBMC depleted $CD4^+$ or $CD8^+$ cells showed that T cells producing IFN-γ against whole AFP consisted of both $CD8^+$ and $CD4^+$ cells (*b*). Assays were performed in 5 patients and the representative result is shown.

Table 2. T cell response to other tumor antigen-derived peptides by ELISPOT assay before and after TAE

	Before treatment						After treatment					
Patient	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$	$MRP3_{503}$	$MRP3_{692}$	$MRP3_{765}$	$hTERT_{167}$	$hTERT_{324}$	$hTERT_{461}$
5	2	7	8	0	3.5	7.5	0	0	0	7	3	35
8	6	6	1	3	ND	ND	**17**	**18**	**22**	**18**	**14**	9
10	0	1	3	0	5	7	0	4	7	6	**11**	4
14	6	5	0	9	5	**13**	6	**14**	**22**	8	**10**	7

Abbreviation: ND, not done. The bold letters show the positive responses in ELISPOT assays.

response with CMV-derived peptide or tetanus toxoid was not different between the 2 groups (Figs. 2*b* and 2*c*).

In the comparison of the mean values of spots generated with AFP-derived peptides, protein, CMV-derived peptides or tetanus toxoid, no significant difference was observed between patients with TAE alone before and after treatment (Figs. 3*a*–3*d*). In contrast, the mean values of spots generated with AFP-derived peptides were significantly higher in patients after TAE with DC infusion than in those before treatment (Fig. 3*e*). The mean values of spots generated with protein, CMV-derived peptides or tetanus toxoid were not significantly different between patients before and after TAE with DC infusion (Figs. 3*f*–3*h*). Based on the above results, we considered that the main difference between TAE alone and TAE with DC infusion was the response to HLA-A24-restricted AFP-derived epitopes. Therefore, to analyze the difference between TAE alone and TAE with DC infusion more precisely, we selected the patients with HLA-A24 or A23 and compared the clinical parameters of both groups. However, there were no statistical differences except liver function in the 2 groups (Table 5).

Enhancement of AFP-specific T-cell responses and treatment outcome

To evaluate the effect of immune enhancement by TAE or TAE with DC infusion for the treatment outcome, we analyzed the clinical course of 17 patients who received complete ablation by additional RFA, PEIT or MCT after these treatments and could be followed up using dynamic CT every 3 months (Table 6). Seven patients showed increasing specific spots for AFP or AFP-derived peptides in ELISPOT assay after TAE. HCC recurrence within 3 months after complete ablation was observed in 3 patients who showed increasing AFP-specific T-cell responses after TAE. Furthermore, recurrence within 6 months after complete ablation was observed