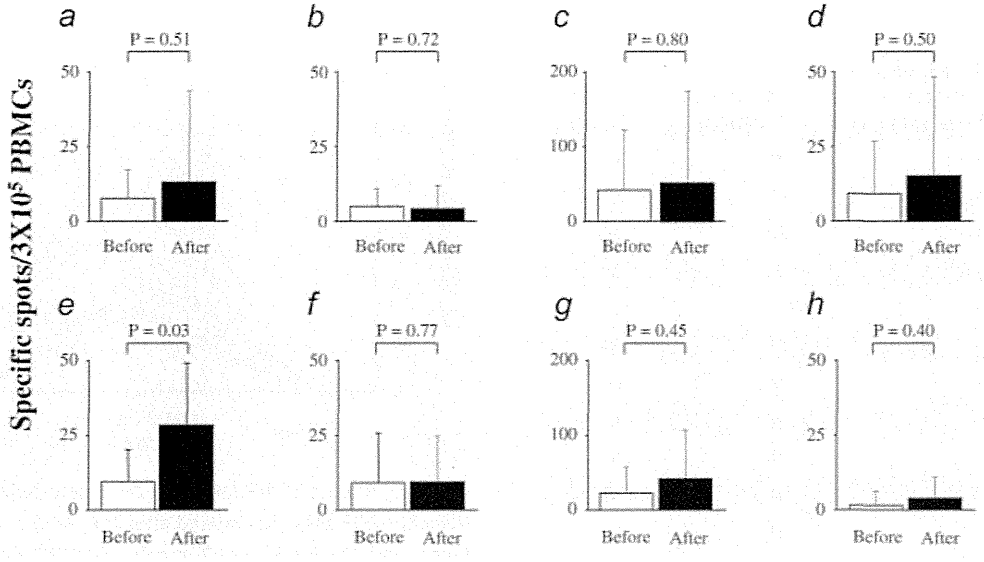


Figure 3. Comparison of direct *ex vivo* analysis (IFN-γ ELISPOT assay) before and after treatment of HCC. The assay was performed using PBMCs of patients who received TAE for AFP-derived peptides (*a*), AFP (*b*), CMV pp65-derived peptide (*c*) or tetanus toxoid protein (*d*). The same assay was performed using PBMCs of patients who received TAE with DC infusion for AFP-derived peptides (*e*), AFP (*f*), CMV pp65-derived peptide (*g*) or tetanus toxoid protein (*h*). AFP and CMV pp65-derived peptides were tested in only HLA-A24 or A23 positive patients. Data are expressed as the mean + SD of specific spots.

Table 5. Characteristics of the patients with HLA-A24 or A23

	Patients treated by TAE (n = 16)	Patients treated by TAE with DC (n = 9)	p-value[1]
Age (years)[2]	65.7 ± 7.8	67.8 ± 10.8	NS
Sex (M/F)	10/6	7/2	NS
ALT (IU/l)	55.9 ± 51.9	75.4 ± 53.0	NS
Total bilirubin (g/dl)	1.4 ± 0.8	1.4 ± 1.1	NS
Albumin (g/dl)	3.6 ± 0.7	3.1 ± 0.6	NS
AFP level (ng/ml)	392.1 ± 877.8	337.2 ± 477.1	NS
Diff. degree of HCC (well/moderate or poor/ND[1])	2/5/9	3/3/3	NS
Tumor size (small/large[3])	3/13	0/9	NS
Tumor multiplicity (multiple/solitary)	15/1	8/1	NS
TNM stage (I, II/III, IV)	15/1	7/2	NS
Histology of nontumor liver (LC/chronic hepatitis)	13/3	8/1	NS
Liver function (Child A/B or C)	10/6	0/9	0.003
Etiology (HCV/HBV/others)	11/1/4	9/0/0	NS

[1]Abbreviations: NS, no statistical significance; ND, not determined. [2]Data are expressed as the mean ± SD. [3]Small: ≤2 cm, large: >2 cm.

The precise mechanism of this phenomenon is still unknown; however, in recent studies, several treatments to destroy tumor cells by necrosis and/or apoptosis have induced antitumor immune responses in animal models[14,44] and even in humans.[6–10] In the study of *in situ* tumor ablation, it is reported that tumor ablation creates a tumor antigen source for the induction of antitumor immunity.[9,44] In another study regarding photodynamic therapy (PDT),[45] it is reported that acute inflammation, expression of heat-shock proteins and providing tumor antigens to DCs caused by PDT induce tumor-specific immune responses.

Based on these results, we hypothesize that DC infusion with TAE can induce antitumor immune responses more effectively than TAE alone. According to DC research in recent years, successful enhancement of the antitumor immune response has been reported by intratumoral

Table 6. Enhancement of AFP-specific T cell response and treatment outcome

	Enhancement of AFP-specific T cell response	Recurrence, 3 months	Recurrence, 6 months
Patient 1	−	N	U
Patient 2	−	N	M
Patient 4	+	M	ND
Patient 5	−	N	M
Patient 6	+	N	U
Patient 9	−	N	M
Patient 10	−	N	N
Patient 13	−	N	N
Patient 14	−	N	N
Patient 16	−	N	M
Patient 19	−	N	U
Patient 24	+	U	ND
Patient 25	+	M	ND
Patient 26	+	N	N
Patient 30	+	N	N
Patient 31	+	N	N
Patient 33	−	N	N

Abbreviations: N, no recurrence; U, uninodular recurrence; M, multinodular recurrence; ND, not determined.

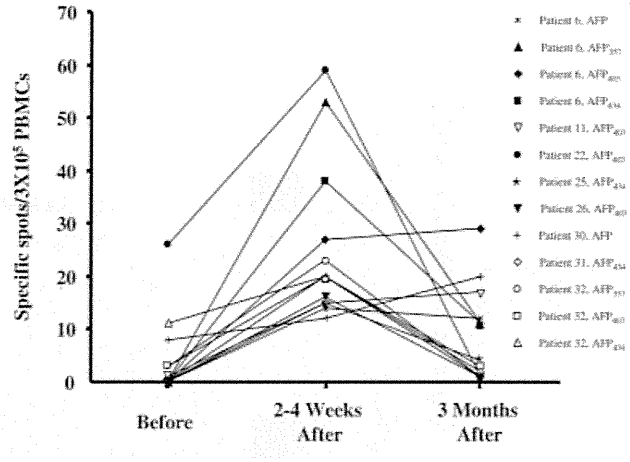


Figure 4. Kinetics of AFP-specific T-cell responses determined by IFN-γ ELISPOT assay before and after TAE. PBMCs were obtained before and 2–4 weeks and 3 months after TAE. Each graph indicates the kinetics of T cells specific for each antigen in each patient. Some patients received additional treatments as indicated in Tables 1 and 3 for a curative treatment after the measurement of T-cell responses at 2–4 weeks after TAE.

administration of DC in combination with tumor ablation.[46,47] Furthermore, immunotherapies using DC have been performed in patients with HCC and their antitumor effects are reported.[48–50] These results support our hypothesis and therefore, in the next step, we examined the immunological effects of DC infusion with TAE.

The comparison of frequency in patients who showed enhancement of AFP-specific immune responses revealed more frequency in patients with DC infusion than in those with TAE alone. On the other hand, there were no differences in the 2 groups in the comparison of frequency for patients who showed enhancement of CMV or TT-specific immune responses. These results suggest that DC infusion with TAE affects tumor-specific immune responses and that the effects are limited to the tumor area.

Some patients with TAE alone showed disappearance of AFP- or control antigen-specific T cells. Although the mechanism of this phenomenon is unknown, anticancer drugs used in TAE might suppress the immune responses, because most of the patients showed decreasing the number of lymphocytes after TAE. These results suggest that TAE alone might give a chance to enhance tumor-specific T-cell responses in only some patients. Further analysis using many more patients with TAE is necessary to make clear the differences in the patients with and without enhancement of T-cell responses. In contrast, disappearance of AFP- or control antigen-specific T cells was not observed in the patients with DC infusion, suggesting strong immunostimulating effect of this treatment.

In analysis of the association between the enhancement of AFP-specific T cells and clinical responses, no correlation could be shown, suggesting that enhancement of T-cell response associated with TAE or TAE with DC infusion may not have protective effect against HCC recurrence. To clarify the mechanism in more detail, we examined the kinetics of AFP-specific T-cell response. Increased frequency of AFP-specific T cells was transient and fell in 4 of 8 patients 3 months after treatment (Fig. 4). Similar to our results, Ayaru *et al.* also reported that the frequency of AFP-specific $CD4^+$ T cells fell in all patients by 1–3 months after TAE.[8] In addition, our results suggest that DC infusion with TAE is not effective to maintain the increased frequency of AFP-specific T cells.

Recent genome profiling studies of HCC show that HCC is a very heterogenous tumor.[51] Furthermore, HCC has multicentric carcinogenesis and develops at different time points. These characters of HCC may also be another reason for no correlation between the enhancement of AFP-specific T cells and clinical responses. The identification of many more tumor antigens and their T-cell epitopes is necessary for more precise analysis of the relationship between antitumor immune response and clinical response, and for immunotherapy.

In the recent study, it is reported that $CD8^+$ T-cell response to AFP is multispecific and AFP-specific IFN-γ-producing $CD8^+$ T cells are directed against different epitopes spreading over the entire AFP sequence with no single

immuno-dominant $CD8^+$ T-cell epitope.[52] Therefore, there is a limitation to our study, because the number of immunogenic AFP-derived peptides applicable in this study is small. However, the results of the present study suggest that TAE with DC infusion enhances the tumor-specific immune responses. Although these modified immune responses may not be sufficient to prevent HCC recurrence because the enhanced immune responses are transient and attenuate within 3 months, these results may contribute to the development of novel immunotherapeutic approach for HCC.

Acknowledgements

The authors thank Ms. Maki Kawamura and Ms. Kazumi Fushimi for technical assistance and for their invaluable help with sample collection.

References

1. Curley SA, Izzo F, Ellis LM, Nicolas Vauthey J, Vallone P. Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. *Ann Surg* 2000;232: 381–91.
2. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996;334: 693–9.
3. Urabe T, Kaneko S, Matsushita E, Unoura M, Kobayashi K. Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-alpha-2b for patients with locally advanced hepatocellular carcinoma. *Oncology* 1998; 55:39–47.
4. Ishizaki Y, Kawasaki S. The evolution of liver transplantation for hepatocellular carcinoma (past, present, and future). *J Gastroenterol* 2008;43:18–26.
5. Okuwaki Y, Nakazawa T, Shibuya A, Ono K, Hidaka H, Watanabe M, Kokubu S, Saigenji K. Intrahepatic distant recurrence after radiofrequency ablation for a single small hepatocellular carcinoma: risk factors and patterns. *J Gastroenterol* 2008;43:71–8.
6. Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, Stern PL, Moore JV, Corbitt G, Kitchener HC, Hampson IN. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. *Cancer Res* 2001;61:192–6.
7. Nakamoto Y, Mizukoshi E, Tsuji H, Sakai Y, Kitahara M, Arai K, Yamashita T, Yokoyama K, Mukaida N, Matsushima K, Matsui O, Kaneko S. Combined therapy of transcatheter hepatic arterial embolization with intratumoral dendritic cell infusion for hepatocellular carcinoma: clinical safety. *Clin Exp Immunol* 2007;147: 296–305.
8. Ayaru L, Pereira SP, Alisa A, Pathan AA, Williams R, Davidson B, Burroughs AK, Meyer T, Behboudi S. Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. *J Immunol* 2007;178:1914–22.
9. Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, Schivazappa S, Zibera C, Fagnoni FF, Ferrari C, Missale G. Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. *Cancer Res* 2006;66:1139–46.
10. 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: 1194–204.
11. Gollnick SO, Evans SS, Baumann H, Owczarczak B, Maier P, Vaughan L, Wang WC, Unger E, Henderson BW. Role of cytokines in photodynamic therapy-induced local and systemic inflammation. *Br J Cancer* 2003;88:1772–9.
12. Gollnick SO, Owczarczak B, Maier P. Photodynamic therapy and anti-tumor immunity. *Lasers Surg Med* 2006;38: 509–15.
13. Yamamoto N, Homma S, Sery TW, Donoso LA, Hoober JK. Photodynamic immunopotentiation: in vitro activation of macrophages by treatment of mouse peritoneal cells with haematoporphyrin derivative and light. *Eur J Cancer* 1991;27: 467–71.
14. den Brok MH, Sutmuller RP, van der Voort R, Bennink EJ, Figdor CG, Ruers TJ, Adema GJ. In situ tumor ablation creates an antigen source for the generation of antitumor immunity. *Cancer Res* 2004;64: 4024–9.
15. Kotera Y, Shimizu K, Mule JJ. Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. *Cancer Res* 2001; 61:8105–9.
16. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* 2000;191:423–34.
17. Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. *Cancer Res* 2005;65: 1018–26.
18. Takayasu K, Arii S, Ikai I, Omata M, Okita K, Ichida T, Matsuyama Y, Nakanuma Y, Kojiro M, Makuuchi M, Yamaoka Y. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology* 2006;131:461–9.
19. Matsui O, Kadoya M, Yoshikawa J, Gabata T, Arai K, Demachi H, Miyayama S, Takashima T, Unoura M, Kogayashi K. Small hepatocellular carcinoma: treatment with subsegmental transcatheter arterial embolization. *Radiology* 1993;188:79–83.
20. Yamada R, Kishi K, Sonomura T, Tsuda M, Nomura S, Satoh M. Transcatheter arterial embolization in unresectable hepatocellular carcinoma. *Cardiovasc Intervent Radiol* 1990;13:135–9.
21. Pelletier G, Roche A, Ink O, Anciaux ML, Derhy S, Rougier P, Lenoir C, Attali P, Etienne JP. A randomized trial of hepatic arterial chemoembolization in patients with unresectable hepatocellular carcinoma. *J Hepatol* 1990;11:181–4.
22. Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire. A comparison of lipiodol chemoembolization and conservative treatment for unresectable hepatocellular carcinoma. *N Engl J Med* 1995;332:1256–61.
23. Bruix J, Llovet JM, Castells A, Montana X, Bru C, Ayuso MC, Vilana R, Rodes J. Transarterial embolization versus symptomatic treatment in patients with advanced hepatocellular carcinoma: results of a randomized, controlled trial in a single institution. *Hepatology* 1998;27:1578–83.
24. Llovet JM, Real MI, Montana X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Sola R, Rodes J, Bruix J. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002;359:1734–9.
25. Lo CM, Ngan H, Tso WK, Liu CL, Lam CM, Poon RT, Fan ST, Wong J. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002;35:1164–71.
26. Hsu HC, Wei TC, Tsang YM, Wu MZ, Lin YH, Chuang SM. Histologic assessment of resected hepatocellular carcinoma after

transcatheter hepatic arterial embolization. *Cancer* 1986;57:1184–91.
27. Kenji J, Hyodo I, Tanimizu M, Tanada M, Nishikawa Y, Hosokawa Y, Mandai K, Moriwaki S. Total necrosis of hepatocellular carcinoma with a combination therapy of arterial infusion of chemotherapeutic lipiodol and transcatheter arterial embolization: report of 14 cases. *Semin Oncol* 1997;24: S6–71–S6–80.
28. Kobayashi N, Ishii M, Ueno Y, Kisara N, Chida N, Iwasaki T, Toyota T. Co-expression of Bcl-2 protein and vascular endothelial growth factor in hepatocellular carcinomas treated by chemoembolization. *Liver* 1999;19:25–31.
29. Xiao EH, Li JQ, Huang JF. Effects of p53 on apoptosis and proliferation of hepatocellular carcinoma cells treated with transcatheter arterial chemoembolization. *World J Gastroenterol* 2004;10:190–4.
30. Kanai M, Kohda H, Sekiya C, Namiki M. Effects on interleukin 1 alpha and beta production of peripheral blood mononuclear cells from patients with hepatocellular carcinoma after transcatheter arterial embolization. *Gastroenterol Jpn* 1990;25:662.
31. Yamazaki H, Nishimoto N, Oi H, Matsushita M, Ogata A, Shima Y, Inoue T, Tang JT, Yoshizaki K, Kishimoto T, Inoue T. Serum interleukin 6 as a predicator of the therapeutic effect and adverse reactions after transcatheter arterial embolization. *Cytokine* 1995;7:191–5.
32. Itoh Y, Okanoue T, Ohnishi N, Nishioji K, Sakamoto S, Nagao Y, Nakamura H, Kirishima T, Kashima K. Hepatic damage induced by transcatheter arterial chemoembolization elevates serum concentrations of macrophage-colony stimulating factor. *Liver* 1999;19:97–103.
33. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. *AJR Am J Roentgenol* 1980;135: 1037–43.
34. Sobin LH, Wittekind C. TNM classification of malignant tumors, 6th edn. New York: Wiley-Liss, 2002. 81.
35. Terayama N, Miyayama S, Tatsu H, Yamamoto T, Toya D, Tanaka N, Mitsui T, Miura S, Fujisawa M, Kifune K, Matsui O, Takashima T. Subsegmental transcatheter arterial embolization for hepatocellular carcinoma in the caudate lobe. *J Vasc Interv Radiol* 1998;9:501–8.
36. Okamoto H, Shin J, Mion S, Koshimura S, Shimizu R. Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci. *Jpn J Microbiol* 1967;11: 323–36.
37. Nakahara S, Tsunoda T, Baba T, Asabe S, Tahara H. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide. *Cancer Res* 2003; 63:4112–8.
38. Japan LCSGo. Classification of primary liver cancer. English edn. 2. Tokyo: Kanehara, 1997.
39. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513–20.
40. Mizukoshi E, Nakamoto Y, Marukawa Y, Arai K, Yamashita T, Tsuji H, Kuzushima K, Takiguchi M, Kaneko S. Cytotoxic T cell responses to human telomerase reverse transcriptase in patients with hepatocellular carcinoma. *Hepatology* 2006;43:1284–94.
41. Mizukoshi E, Honda M, Arai K, Yamashita T, Nakamoto Y, Kaneko S. Expression of multidrug resistance-associated protein 3 and cytotoxic T cell responses in patients with hepatocellular carcinoma. *J Hepatol* 2008;49:946–54.
42. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M. 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: 6242–52.
43. 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;98:1872–81.
44. Wissniowski TT, Hansler J, Neureiter D, Frieser M, Schaber S, Esslinger B, Voll R, Strobel D, Hahn EG, Schuppan D. Activation of tumor-specific T lymphocytes by radio-frequency ablation of the VX2 hepatoma in rabbits. *Cancer Res* 2003;63: 6496–500.
45. Korbelik M, Krosl G, Krosl J, Dougherty GJ. The role of host lymphoid populations in the response of mouse EMT6 tumor to photodynamic therapy. *Cancer Res* 1996;56: 5647–52.
46. Udagawa M, Kudo-Saito C, Hasegawa G, Yano K, Yamamoto A, Yaguchi M, Toda M, Azuma I, Iwai T, Kawakami Y. Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. *Clin Cancer Res* 2006;12: 7465–75.
47. Machlenkin A, Goldberger O, Tirosh B, Paz A, Volovitz I, Bar-Haim E, Lee SH, Vadai E, Tzehoval E, Eisenbach L. Combined dendritic cell cryotherapy of tumor induces systemic antimetastatic immunity. *Clin Cancer Res* 2005;11: 4955–61.
48. Ladhams A, Schmidt C, Sing G, Butterworth L, Fielding G, Tesar P, Strong R, Leggett B, Powell L, Maddern G, Ellem K, Cooksley G. Treatment of non-resectable hepatocellular carcinoma with autologous tumor-pulsed dendritic cells. *J Gastroenterol Hepatol* 2002;17: 889–96.
49. Iwashita Y, Tahara K, Goto S, Sasaki A, Kai S, Seike M, Chen CL, Kawano K, Kitano S. A phase I study of autologous dendritic cell-based immunotherapy for patients with unresectable primary liver cancer. *Cancer Immunol Immunother* 2003; 52:155–61.
50. Lee WC, Wang HC, Hung CF, Huang PF, Lia CR, Chen MF. Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. *J Immunother* 2005;28: 496–504.
51. Lee JS, Thorgeirsson SS. Genome-scale profiling of gene expression in hepatocellular carcinoma: classification, survival prediction, and identification of therapeutic targets. *Gastroenterology* 2004; 127:S51–5.
52. Thimme R, Neagu M, Boettler T, Neumann-Haefelin C, Kersting N, Geissler M, Makowiec F, Obermaier R, Hopt UT, Blum HE, Spangenberg HC. Comprehensive analysis of the alpha-fetoprotein-specific CD8+ T cell responses in patients with hepatocellular carcinoma. *Hepatology* 2008;48:1821–33.

Clin Orthop Relat Res (2010) 468:1373–1383
DOI 10.1007/s11999-010-1302-z

BASIC RESEARCH

Cryoimmunologic Antitumor Effects Enhanced by Dendritic Cells in Osteosarcoma

Masanori Kawano MD, Hideji Nishida MD, PhD, Yasunari Nakamoto MD, PhD, Hiroshi Tsumura MD, PhD, Hiroyuki Tsuchiya MD, PhD

Received: 20 February 2009 / Accepted: 1 March 2010 / Published online: 16 March 2010

Abstract

Background We previously reported a limb-salvage technique by treating tumor-bearing bone with liquid nitrogen. We also reported systemic antitumor immunity was enhanced by cryotreatment in a murine osteosarcoma (LM8) model. We therefore combined the cryotreatment of tumor with dendritic cells to promote tumor-specific immune responses.

Questions/purposes We determined whether our technique could enhance systemic immune response and inhibit metastatic tumor growth in a murine osteosarcoma model.

Materials and Methods To evaluate activation of the immune response, we prepared six groups of C3H mice (80 mice total): (1) excision only, (2) dendritic cells without reimplantation of the cryotreated primary tumor, (3) reimplantation of the cryotreated primary tumor alone, (4) dendritic cells combined with reimplantation of the cryotreated primary tumor, (5) dendritic cells exposed to cryotreated tumor lysates without reimplantation of the cryotreated primary tumor, and (6) dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor. We then compared and verified the activation state of each group's antitumor immunity.

Results Mice that received dendritic cells exposed to cryotreated tumor lysates with reimplantation of the cryotreated primary tumor group had high serum interferon γ, reduced pulmonary metastases, and increased numbers of CD8(+) T lymphocytes in the metastatic areas.

Conclusions Combining tumor cryotreatment with dendritic cells enhanced systemic immune responses and inhibited metastatic tumor growth.

Clinical Relevance We suggest immunotherapy could be developed further to improve the treatment of osteosarcoma.

Each author certifies that he or she has no commercial associations that might pose a conflict of interest in connection with the submitted article.
Each author certifies that his or her institution has approved the animal protocol for this investigation, and that all investigations were conducted in conformity with ethical principles of research.
This work was performed at the Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, and the Department of Orthopaedic Surgery, Faculty of Medicine, Oita University.

M. Kawano, H. Nishida, H. Tsuchiya (✉)
Department of Orthopaedic Surgery, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan
e-mail: tsuchi@med.kanazawa-u.ac.jp

M. Kawano, H. Tsumura
Department of Orthopaedics Surgery, Faculty of Medicine, Oita University, Oita, Japan

Y. Nakamoto
Department of Gastroenterology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

Introduction

The standard treatment of osteosarcoma consists of preoperative chemotherapy, surgical tumor excision, and postoperative chemotherapy. Limb-saving surgery is feasible in most cases. Advances in osteosarcoma treatment have now achieved a 5-year survival rate of 60% to 90% for patients, and limb function after reconstruction continues to improve with time [3, 16, 30, 46, 47, 49].

Tsuchiya et al. developed a new approach using frozen autografts [48] to improve reconstruction after osteosarcoma resection. The tumor is resected with an adequate margin, and the resected specimen is immersed in liquid

nitrogen for 20 minutes to kill all tumor cells. After thawing, the specimen is returned to the original place with appropriate internal fixation to reconstruct the defect. Compared with heat-treated bones [8, 14], bone genetic proteins and native biomechanical structures are preserved after cryotreatment [53]. In one report limb function using the technique of Tsuchiya et al. was rated as excellent in 71.4% of patients, and good in 10.7%, as assessed by the functional evaluation system of Enneking [11]. Two studies suggest the approach enhanced bone formation when compared histologically with pasteurized bone and irradiated bone [43, 48]. Another advantage in reimplanting cryotreated tumor tissue is its effect on the immune system [50]: tumor tissue after cryoablation in situ provokes an immune reaction in patients with breast and prostate cancer [6, 8, 39]. Brewer et al. reported metastatic tumors sometimes disappear or shrink after in situ cryoablation of the primary tumor with liquid nitrogen [4]. The structure of tumor antigens is retained in frozen tumor, and leukocytes probably can recognize these antigens. Similar antitumor effects can be expected from our reconstructive procedure of reimplanting tumor-bearing bone after cryotreatment with liquid nitrogen.

Nishida et al. observed an inadequate antitumor effect after reimplantation of frozen tumor tissue alone [35]. However, the antitumor effect was enhanced by promoting nonspecific immune activation by intraperitoneal injection of OK-432, a substance extracted from alpha-Streptococcus pyogenes. This approach promotes inflammation and activation of dendritic cells (DCs) that initiate the specific antitumor effect [19]. This type of immunotherapy reportedly is effective for breast and prostate cancers [6, 8, 39]. Many groups have reported successful immunotherapy for osteosarcoma [5, 15, 18, 20, 22, 24, 25, 33, 34, 36, 42, 51, 52]. However, the ability to control metastatic lesions and local recurrence does not appear to be superior to other adjuvant treatments [2, 7, 13, 23, 29].

We therefore wondered whether combining cryotreatment and immunotherapy might enhance tumor response. We specifically determined whether: (1) antitumor immunity could be enhanced through activation and transfer of DCs combined with reimplantation of the cryotreated primary tumor, and (2) metastatic lesions could be prevented owing to the involvement of T lymphocytes in a murine osteosarcoma model (LM8).

Materials and Methods

Using a reported method to induce osteosarcoma [1, 35], we hypodermically implanted 1×10^6 LM8 cells (a murine osteosarcoma cell line) into the subcutaneous gluteal region of 80 female C3H mice, 6 to 8 weeks old. Tumors developed in all animals. Two weeks after inoculation, we surgically excised the tumors and cryotreated them with liquid nitrogen. We established the following six groups (Fig. 1): (1) the tumor was excised with wide margins 14 days after inoculation (n = 15); (2) the tumor was excised with wide margins 14 days after inoculation and bone marrow-derived DCs then were injected into the contralateral subcutaneous gluteal region without reimplantation of the cryotreated primary tumor twice a week (n = 15); (3) the tumor was excised with wide margins 14 days after inoculation and reimplanted after cryotreatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence from frozen tumor tissue (n = 15); (4) the tumor was excised 14 days after inoculation and reimplanted after cryotreatment into the contralateral gluteal region to evaluate for local recurrence, and DCs then were injected twice a week into this secondary site (n = 15); (5) the tumor was excised with wide margins 14 days after inoculation and DCs exposed to cryotreated tumor lysates were injected twice a week into the contralateral gluteal region without reimplantation of the cryotreated primary tumor (n = 15); and (6) the tumor was excised with wide margins 14 days after inoculation and reimplanted after the treatment with liquid nitrogen into the contralateral gluteal region to evaluate for local recurrence (same as Group 3) with the addition of DCs exposed to cryotreated tumor lysates injected twice a week (n = 15). We harvested tumor from 30 mice, and then the tumor was treated with liquid nitrogen to create the lysates. We presumed a systemic immune response would be induced by injecting DCs around the frozen tumor tissue. We microscopically determined the presence of metastases in the lungs 2 weeks after the tumor inoculation. We had previously confirmed the presence of pulmonary metastases in an additional 20 mice in a preliminary experiment in advance. We also confirmed that there were no viable cells after cryotreatment using liquid nitrogen, in agreement with a previous study [35]. We observed no recurrence of the tumor at the primary site of inoculation after excision. All experiments were performed under the guidelines for animal experiments as stipulated by the Kanazawa University Graduate School of Medical Science [37].

LM8 cells, derived from Dunn osteosarcoma, were provided by the Riken BioResource Center (Saitama, Japan). The cells were maintained in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 μg streptomycin per mL, and 100 units penicillin per mL and were cultured at 37°C in 5% CO_2. To establish local implantation of the tumor and subsequent lung metastasis, the LM8 cells (1×10^6) were suspended in 0.2 mL phosphate-buffered saline (PBS) and subcutaneously inoculated into the right

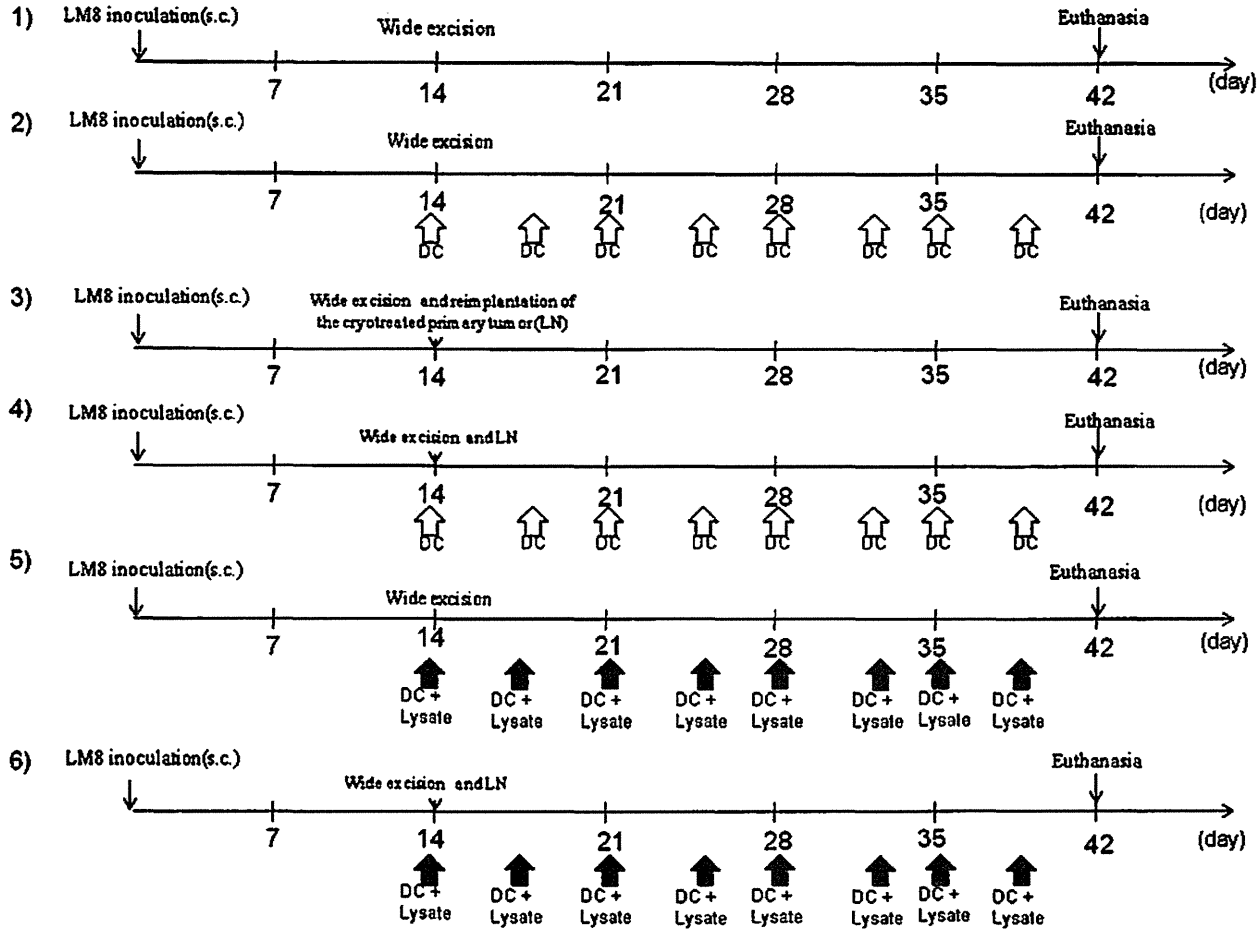


Fig. 1 A diagram of the experimental protocol and treatment schedule is shown. Two weeks after tumor inoculation, tumors were treated by one of the following methods: (1) excision only (n = 15); (2) DCs without reimplantation of the cryotreated primary tumor (n = 15); (3) reimplantation of the cryotreated primary tumor (n = 15); (4) DCs pulsed with cryotreated tumor lysates and reimplantation of the cryotreated primary tumor (n = 15); (5) DCs pulsed with cryotreated tumor lysates without reimplantation of the cryotreated primary tumor (n = 15); or (6) DCs pulsed with cryotreated tumor and reimplantation of the cryotreated primary tumor (LN) (n = 15). The mice were euthanized and evaluated 6 weeks after tumor inoculation. sc = subcutaneous.

gluteal region of the mice. All animals had macroscopically and microscopically confirmed lung metastases within 4 weeks [1].

C3H mice were purchased from Sankyo Labo Inc (Toyama, Japan) and housed in a specific pathogen-free animal facility in our laboratory. We were not able to accurately determine the survival time of each group because the guidelines for animal experiments concerning pain required euthanasia in distressed animals.

Liquid nitrogen (−196°C) was used for cryotreatment. Tumor tissue was collected on gauze and soaked in liquid nitrogen for 20 minutes for en bloc tumor tissue freezing. The tumor was prethawed at room temperature (20°C) for 15 minutes and then thawed in distilled water (20°C) for 15 minutes. The liquid nitrogen-treated tumor tissue was transplanted subcutaneously in the left gluteal region of the same mouse.

Because the mice were genetically identical, the structure of the major histocompatibility complex (MHC) Class I molecules was such that the T cells would be able to recognize the MHC Class I with antigens on the antigen-presenting cells (APCs) [17, 27]. Bone marrow-derived DCs were generated as described by Lutz and Rössner [28] with minor modifications. Briefly, erythrocyte-depleted mouse bone marrow cells obtained from flushed marrow cavities (1×10^6 cells/mL) were cultured in complete medium with 20 ng/mL recombinant mouse GMCSF (PeproTech EC Ltd, London, UK) in 10-cm tissue culture dishes at 37°C in an atmosphere containing 50 mL CO_2 per L. On Days 3 and 6, half of the medium was added to the

same volume of fresh complete medium and used to replenish the original plates. The freeze-thawed tumor lysate was added to the DC cultures on Day 6 at a ratio of five DC equivalents to one tumor cell (ie, 5:1) and incubated at 37°C in an atmosphere containing 50 mL CO_2 per L. After 24 hours of incubation, nonadherent cells including DCs were harvested by gentle pipetting.

For fluorescence activated cell sorting (FACS) analysis, DCs were counted with a FACSCalibur™ Flow Cytometer (Becton-Dickinson, San Jose, CA) and stained with fluorochrome-conjugated antibodies (BD Pharmingen, Tokyo, Japan) for the following markers: cluster of differentiation (CD)11c, CD80, CD86, I-Ad, and CD40. CD11c was used as a marker for all DCs regardless of the degree of maturation, whereas CD80, CD86, I-Ad, and CD40 are markers for DCs. Data analysis was performed with CELLQuest™ software (Becton-Dickinson). The corresponding labeled isotype antibodies served as controls. DCs used for vaccination were washed twice, enumerated, and resuspended in PBS at 1×10^6/mL.

We inoculated LM8 cells (5×10^6) in a mouse to make the tumor lysate. After 4 weeks, we resected the tumor mass and soaked the entire tumor in liquid nitrogen to kill the tumor cells. We mixed cryonecrotic tissue with DCs at Culture Day 6, after the tumor was defrosted, and the homogenate was prepared using PBS. The homogenate was passed through a 0.2-μm filter to remove bacteria and tissues and mixed with the DCs for 24 hours.

After intraperitoneal injection of 5 mL sodium pentobarbital (Somnopentyl®; Kyontsu Seiyaku, Tokyo, Japan), mice were euthanized by cervical dislocation and their blood was collected. Murine interferon (IFN)-γ and interleukin (IL)-4 release were measured by ELISA using Quantikine® (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol using an Easy Reader EAR340 microtest plate reader (SLT-Labinstruments, Salzburg, Austria).

We estimated the area of the pulmonary metastatic lesion on 50 serial histologic sections of each lung by manually drawing orthogonal lines delimiting the edges of the pulmonary metastatic lesion and selected the widest part of the specimen. The area was determined by multiplying the maximum orthogonal dimensions using ImageJ software (NIH, Bethesda, MD; http://rsb.info.nih.gov/ij/). We compared the mean areas between the six groups.

For immunohistochemistry, lung specimens were fixed in 20% formalin and embedded in paraffin. For each case, we examined all the blocks of lung tissues of formalin-fixed, paraffin-embedded tumor tissue. All specimens were decalcified, although we found the decalcification step did not influence the immunohistochemistry for any of the stains. Five sections for each mouse were cut 4-μm thick. Each section was cut at the maximum diameter. CD8(+) T lymphocytes and natural killer (NK) cells in the pulmonary metastatic lesion were quantified by measuring the immunohistochemistry-positive cells per unit area in each group. Rehydrated tissue sections were incubated with rat monoclonal antibody raised against CD8(+) T lymphocytes of mouse origin (Santa Cruz Biotechnology, Santa Cruz, CA) and rat monoclonal antibody raised against NK cells of mouse origin (Abcam Plc, Cambridge, UK). The two antibodies were diluted 1:50 with PBS. Color reactions were performed at room temperature for 15 minutes and cover slips were mounted with glycerol and gelatin.

We determined differences in serum IFN-γ, serum IL-4, pulmonary metastatic area, and number of CD8(+) lymphocytes and NK cells in the metastatic area among the six groups using a nonrepeated-measures ANOVA and the Scheffe test. All analyses were conducted with SPSS® 11.0 software (SPSS Japan Inc, Tokyo, Japan).

Results

We activated antitumor immunity by combining DCs exposed to lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor. On Culture Day 7, the ratio of mature DCs to immature DCs was increased compared with the ratio at Culture Day 6 (Fig. 2; immature DCs, upper left; mature DCs, upper right). Moreover, this increase was more apparent in groups incubated with tumor lysate. Serum IFN-γ levels were greater ($p < 0.0001$) in the mice that received DCs combined with reimplantation of the cryotreated primary tumor (119.0 ± 7.61 pg/mL) than in the cryotreated primary tumor alone group (37.33 ± 2.58 pg/mL). Moreover, the group that received tumor lysate-exposed DCs combined with reimplantation of the cryotreated primary tumor (157.33 ± 14 pg/mL) had a greater ($p < 0.0001$) IFN-γ level than the group that received only tumor lysate-exposed DCs without reimplantation of the cryotreated primary tumor (120.27 ± 11.29 pg/mL) (Fig. 3). Serum IL-4 was lower ($p < 0.0001$) in the mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group (13.33 ± 9.75 pg/mL) than in the excision-only group (45.06 ± 5.71 pg/mL) (Fig. 4).

The enhanced immune response by T lymphocytes reduced metastatic lesions. Reduction of the metastatic area was greater ($p < 0.0001$) in the group that received DCs without reimplantation of the cryotreated primary tumor (15.99 ± 3.93 mm^2) than in the excision-only group (24.12 ± 3.60 mm^2). The reduction of the metastatic area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (5.39 ± 1.49 mm^2) than in the reimplantation of

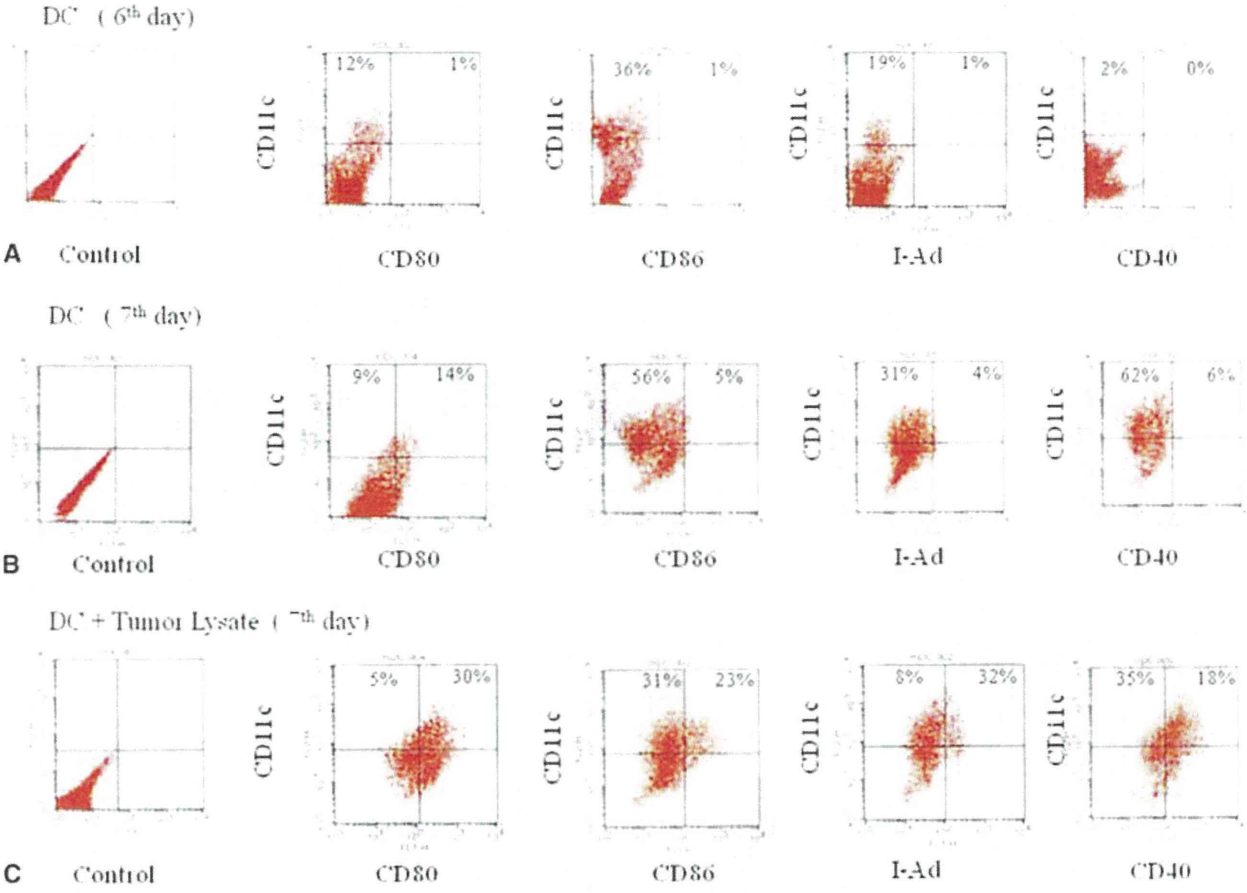
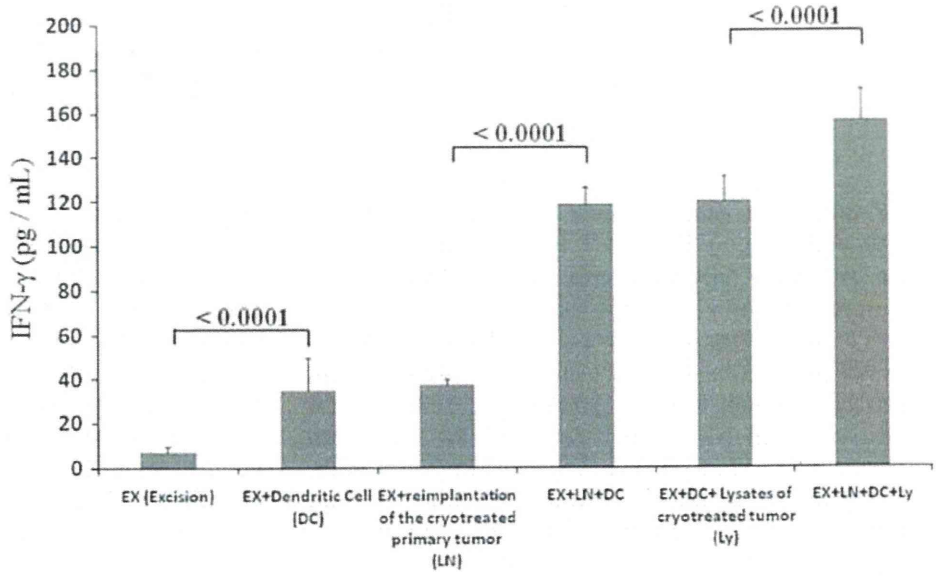


Fig. 2A–C DC activation status was examined using flow cytometry. DCs at Culture Day 7 (Group **B**) were more mature than DCs at Culture Day 6 (Group **A**). On Culture Day 7, DC maturity was greatest in the groups receiving lysate-primed DCs (Group **C**) than in those not receiving lysate-primed DCs (Group **B**).

Fig. 3 A graph of the serum IFN-γ levels in the six treatment groups is shown. The samples were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a highest IFN-γ level. Error bars represent SD.



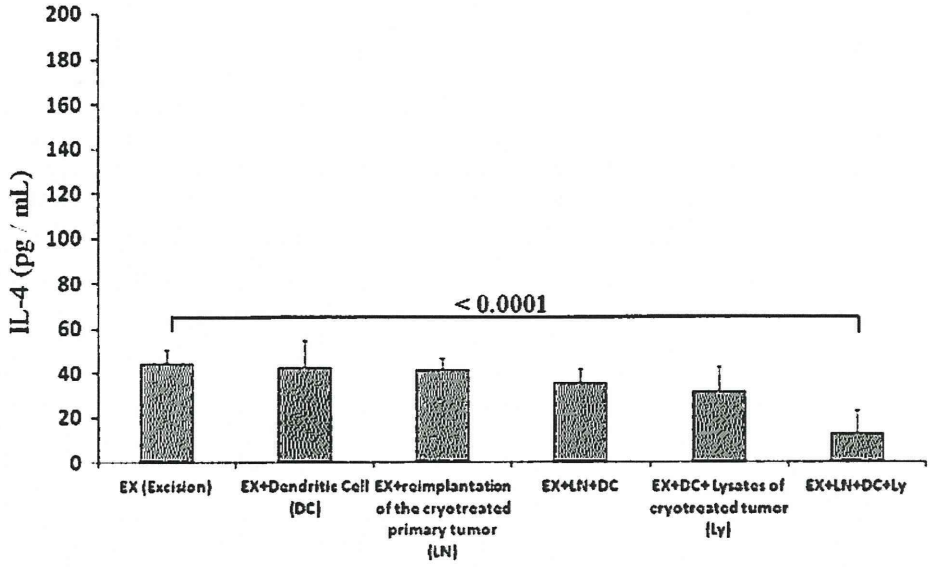


Fig. 4 A graph of the serum IL-4 in the six treatment groups is shown. Sera were collected 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a lower level than any of the other groups. Error bars represent SD.

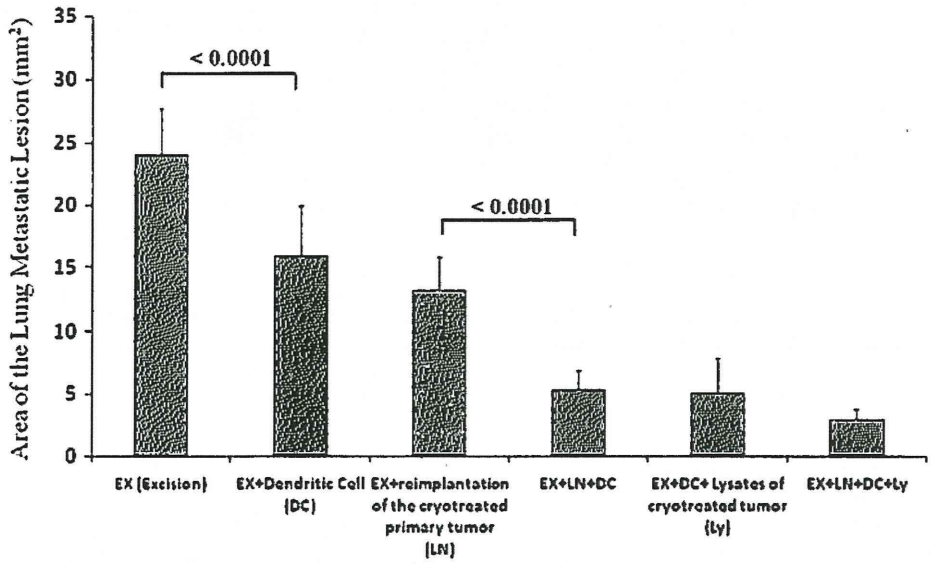


Fig. 5 Reduction of the metastatic area in the six treatment groups is shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

the cryotreated primary tumor alone group (13.22 ± 2.59 mm^2) (Fig. 5). CD8(+) T lymphocytes gathered in the pulmonary metastatic area in DC-treated groups, however, NK cells were not recruited to the metastatic area in the DC-treated groups compared with the nonDC-treated groups (Fig. 6). The number of CD8(+) T lymphocytes per unit area was greater ($p < 0.0001$) in the DCs combined with reimplantation of the cryotreated primary tumor group (8.33 ± 2.57 cells/mm^2) than in the reimplantation of the cryotreated primary tumor alone group (2.44 ± 0.53 cells/mm^2). Mice that received DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor (12.79 ± 2.14 cells/mm^2) showed higher ($p < 0.0001$) levels than the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (8.71 ± 2.39 cells/mm^2) (Fig. 7). The number of NK cells per unit area was greater ($p < 0.0001$) in the group that received DCs exposed to the lysates of cryotreated tumor without reimplantation of the cryotreated primary tumor (3.90 ± 2.17 cells/mm^2) than in the excision-only group (1.20 ± 0.30 cells/mm^2) (Fig. 8). The CD8(+)T lymphocyte, CD4(+) T lymphocyte, and DC infiltrations in reimplanted tumors were similar to those seen with pulmonary metastases (data not shown).

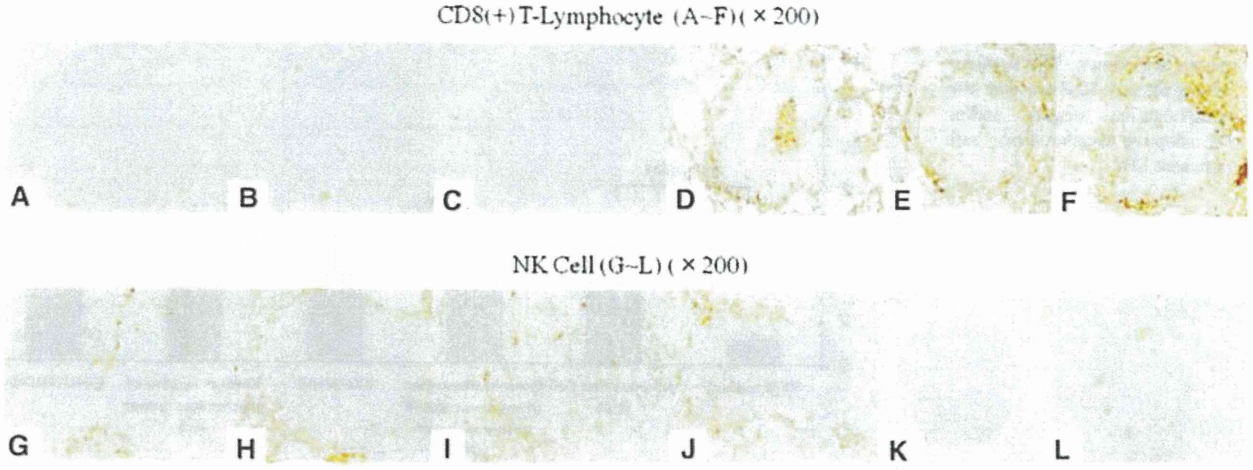
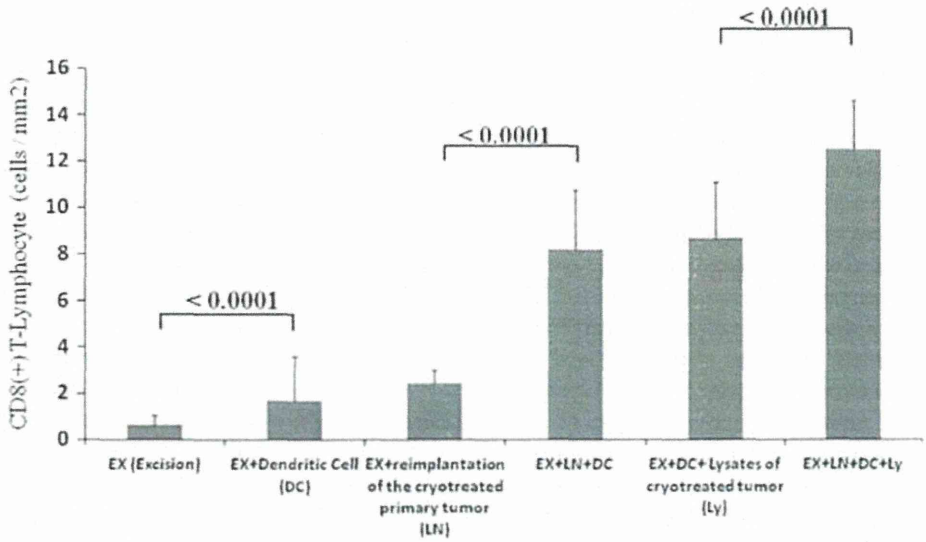


Fig. 6A–L To evaluate CD8(+) T lymphocytes and NK cells in pulmonary metastasis, immunostaining was performed: (**A**) CD8(+) T lymphocytes in Group 1, (**B**) CD8(+) T lymphocytes in Group 2, (**C**) CD8(+) T lymphocytes in Group 3, (**D**) CD8(+) T lymphocytes in Group 4, (**E**) CD8(+) T lymphocytes in Group 5, (**F**) CD8(+) T lymphocytes in Group 6, (**G**) NK cells in Group 1, (**H**) NK cells in Group 2, (**I**) NK cells in Group 3, (**J**) NK cells in Group 4, (**K**) NK cells in Group 5, and (**L**) NK cells in Group 6. CD8(+) T lymphocytes gathered in Groups D,E, and F. However, they did not gather in Groups A, B, and C. However, NK cells were recruited only in Groups A, B, and C. (Original magnification, ×200).

Fig. 7 The numbers of CD8(+) T lymphocytes per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. DCs exposed to the lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor group showed a higher level than any other groups. Error bars represent SD.



Discussion

Various immunotherapies for osteosarcoma have been tried. As standard treatments for osteosarcoma are ineffectual for many patients, new treatments need to be developed. In the 1970s, immunotherapy for osteosarcoma was reported by Southam et al. [42], Neff and Enneking [34], and Campbell et al. [5]. In the 1980s, new methods such as the use of interferons and Bacille de Calmette et Guérin were reported [22, 24, 36]. Another approach used antiidiotypic antibodies using T cells and liposome encapsulation [18, 51, 52]. Current methods of immunotherapy for osteosarcoma include peptide therapy or gene transfer therapy combined with hyperthermia therapy [10, 15, 21, 25, 33]. We asked whether (1) antitumor immunity could be achieved through activation of DCs combined with reimplantation of the cryotreated primary tumor and (2) if metastatic lesions would be prevented owing to enhanced T lymphocyte involvement.

We acknowledge limitations in this study. First, we used mice with an identical genetic makeup. The structure of the MHC Class I molecules was similar and the T cells could recognize the MHC Class I. However, we needed to use DCs from a different (albeit genetically identical) mouse to

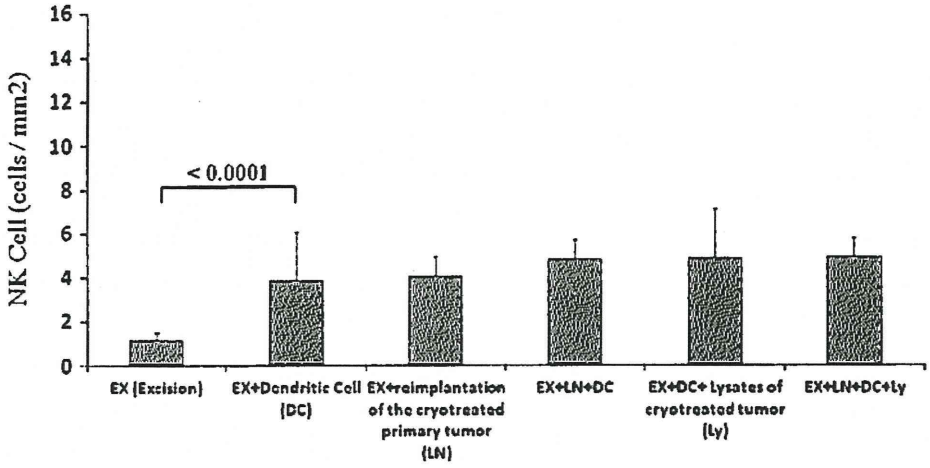


Fig. 8 The numbers of NK cells per unit area in the six treatment groups are shown. The samples were gathered 28 days after the reimplantation surgery and/or DC adoptive transfer. Error bars represent SD.

accomplish our adoptive transfer experiments. We minimized the potential for an immune response to nonself antigens by using genetically identical tumor tissue and mice. It would be necessary to use DCs derived from the same individual in clinical application, but this could not be achieved in our mouse model. In humans, however, monocytes are separated from the patient's own peripheral blood and DCs can be induced from these monocytes. Second, we could not completely replicate the clinical approach used in humans in our mouse model. In clinical cases frozen bone always is returned to the same site. However, it was impossible to replicate this in our experimental mouse model in which transplanted tumor cells were removed from the tibia and then returned to the same place after cryotreatment. In a preliminary experiment we attempted to do just that and these 20 mice could not move and died of starvation. We therefore used the contralateral gluteal region to check for local recurrence after tumor excision or recurrence from frozen tissue.

Antitumor immunity appeared to be activated through DCs combined with reimplantation of the cryotreated primary tumor or by exposing the transferred DC to lysates of cryotreated tumor. The use of lymphokine-activated killer (LAK) therapy has been used with other types of tumors [26]. However, T lymphocytes, which are the effectors, do not accumulate inside osteosarcoma tumors as expected. Autoclaving supplemented by DCs is thought to enhance the antitumor effect, but hyperthermia causes proteins to denature, and activation of the antitumor effect is often insufficient [37]. Several studies [12, 31, 41] report peptide vaccine therapy, but many patients apparently develop immunotolerance [45]. Thus, immunotherapy for malignant tumor achieved by these various methods has not been established definitively although investigations continue to try to overcome the major hurdles associated with immunotherapy (Table 1). We emphasize the immune response is activated by cryotreatment but not by heat-treated tissue. Our method differs from those described by others [7, 9, 10, 14]. In some regards DCs are believed to be the principal APCs for initiating immune responses in vivo [32]. In comparison with other traditional adjunct therapeutic options for cancer, such as radiation therapy and chemotherapy, immunotherapy provides a more targeted treatment to the cancer, with potentially fewer detrimental effects on noncancerous cells [30, 40]. DCs without sufficient cancer antigens may not have the ability to kill tumor cells and present the antigen to T lymphocytes by themselves. Our data suggest the antitumor effect in the group that received DCs without reimplantation of cryotreated primary tumor was almost the same as that in the reimplantation of cryotreated primary tumor alone group. The data further suggest the effects increased only when exposing the DCs to tumor lysates in the absence of cryonecrotic primary tumors. However, combining reimplantation of cryotreated primary tumor and DCs exposed to cryotreated tumor lysates produced synergistic effects. Using reimplantation of cryotreated primary tumor is more appropriate for clinical applications. We therefore believe an efficient immune response will be activated when DCs recognize tumor antigens appropriately. CD8(+) T cells act as an effector by the Th1 route, and this is promoted mainly by IFN-γ and IL-12 [38]. However, IL-4 [21], IL-6, and IL-10 strengthen humoral immunity. Levels of IFN-γ, IL-2, and IL-12 generally increase when cell-mediated immunity is activated, and IL-4, IL-6, and IL-10 increase when humoral immunity is activated. These cytokines act in opposition to maintain an immune balance.

Our data suggest enhanced T lymphocyte recruitment and function reduce metastatic lesions in a murine osteosarcoma model. Immunoreactivity increased slightly in mice that received DCs exposed to lysates of cryotreated tumor combined with reimplantation of the cryotreated primary tumor. NK cells attack the tumor independently of APCs. NK cells attack cells that downregulate MHC Class

Table 1. Immunotherapeutic trials of malignant tumors

Tumor	Immune intervention	Route	Immunologic response	Comments	References
Osteosarcoma	BCG	SC	NC	No consistent clinical effect	[22, 24]
Osteosarcoma	Interferon α	SC, IV	PR–NC	Osteosarcoma-associated antigens have potential for targeted immunotherapy	[36]
Unknown	LAK	IV	NC	T lymphocytes were unable to penetrate the tumor	[26]
Osteosarcoma	Antiidiotypic antibodies	IV	NC	It may be possible to circumvent this heterogeneity by activation of tissue macrophages to the tumoricidal state	[18, 51, 52]
Breast cancer, osteosarcoma	Peptide therapy combined with hyperthermia therapy	SC, IV	NC	It may be a potential agent for use in immunotherapy	[15, 20]
Osteosarcoma	Gene transfer therapy combined with hyperthermia therapy	IV	NC	IL-23 seems to be a less effective immunotherapeutic for adjuvant treatment of osteosarcomas	[25, 33]
Unknown	Peptide vaccine therapy	SC	NC–PD	Many patients have peptide-induced tolerance develop	[45]
Osteosarcoma	Cryoimmunology and DCs	SC	PR	Combining cryotreatment with DCs resulted in enhanced antitumor effects	Our data

BCG = Bacille de Calmette et Guérin; SC = subcutaneous; NC = no change; IV = intravenous; PR = partial response; LAK = lymphokine-activated killer; IL = interleukin; PD = progressive disease; DCs = dendritic cells.

I expression or have a stressed appearance [44]. We observed a reduced tumor burden in the groups that received transplanted DCs, which correlated with recruitment of CD8 lymphocytes to the tumor site as observed with immunohistochemistry.

Returning the frozen bone after liquid nitrogen treatment to its original place can be readily used in the clinic. After the first cryotreatment, it is possible to perform the treatment again using cultured DCs if a patient's tumor cells have been preserved. This approach therefore still can be used even after other methods (such as chemotherapy, radiation therapy, or surgery) no longer are reasonable. Combining DCs pulsed with lysates of cryotreated tumor and reimplantation of the cryotreated primary tumor enhanced antitumor effects. We believe the approach may be a useful alternative for patients with osteosarcoma when other treatment options including chemotherapy, radiotherapy, and surgical treatment have been ineffective.

Acknowledgments We thank Katsuro Tomita, Akihiko Takeuchi, Shuichi Kaneko, and Yohei Marukawa for supervision in this study.

References

1. Asai T, Ueda T, Itoh K, Yoshioka K, Aoki Y, Mori S, Yoshikawa H. Establishment and characterization of a murine osteosarcoma cell line (LM8) with high metastatic potential to the lung. *Int J Cancer.* 1998;76:418–422.
2. Bacci G, Lari S. Adjuvant and neoadjuvant chemotherapy in osteosarcoma. *Chir Organi Mov.* 2001;86:253–268.
3. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, Zoubek A, Jürgens H, Winkler K. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. *J Clin Oncol.* 2002;20:776–790.
4. Brewer WH, Austin RS, Capps GW, Neifeld JP. Intraoperative monitoring and postoperative imaging of hepatic cryosurgery. *Semin Surg Oncol.* 1998;14:129–155.
5. Campbell CJ, Cohen J, Enneking WF. Editorial: New therapies for osteogenic sarcoma. *J Bone Joint Surg Am.* 1975;57:143–144.
6. Chin JL, Lim D, Abdelhady M. Review of primary and salvage cryo-ablation for prostate cancer. *Cancer Control.* 2007;14:231–237.
7. DeLaney TF, Park L, Goldberg SI, Hug EB, Liebsch NJ, Munzenrider JE, Suit HD. Radiotherapy for local control of osteosarcoma. *Int J Radiat Oncol Biol Phys.* 2005;61:492–498.
8. de Moraes AM, Pavarin LB, Herreros F, de Aguiar Michelman F, Velho PE, de Souza EM. Cryosurgical treatment of lentigo maligna. *J Dtsch Dermatol Ges.* 2007;5:477–480.
9. Dinçbaş FO, Koca S, Mandel NM, Hiz M, Dervişoğlu S, Seçmezacar H, Oksüz DC, Ceylaner B, Uzel B. The role of preoperative radiotherapy in nonmetastatic high-grade osteosarcoma of the extremities for limb-sparing surgery. *Int J Radiat Oncol Biol Phys.* 2005;62:820–828.
10. Duparc J, Massin P, Bocquet L, Benfrech E, Cavagna R. [Autoclaved tumoral autografts: apropos of 12 cases, 6 of which highly malignant] [in French]. *Rev Chir Orthop Reparatrice Appar Mot.* 1993;79:261–271.
11. Enneking WF. A system for functional evaluation of the surgical management of musculoskeletal tumors. In: Enneking WF, ed. *Limb Salvage in Musculoskeletal Oncology.* New York, NY: Churchill-Livingstone; 1987:5–16.
12. Enomoto Y, Bharti A, Khaleque AA, Song B, Liu C, Apostolopoulos V, Xing PX, Calderwood SK, Gong J. Enhanced immunogenicity of heat shock protein 70 peptide complexes from

dendritic cell-tumor fusion cells. *J Immunol.* 2006;177:5946–5955.
13. Fagioli F, Biasin E, Mereuta OM, Muraro M, Luksch R, Ferrari S, Aglietta M, Madon E. Poor prognosis osteosarcoma: new therapeutic approach. *Bone Marrow Transplant.* 2008;41(suppl 2): S131–S134.
14. Harrington KD. The use of hemipelvic allografts or autoclaved grafts for reconstruction after wide resections of malignant tumors of the pelvis. *J Bone Joint Surg Am.* 1992;74:331–341.
15. Herbert LM, Grosso JF, Dorsey M Jr, Fu T, Keydar I, Cejas MA, Wreschner DH, Smorodinski N, Lopez DM. A unique mucin immunoenhancing peptide with antitumor properties. *Cancer Res.* 2004;64:8077–8084.
16. Hugate RR, Wilkins RM, Kelly CM, Madsen W, Hinshaw I, Camozzi AB. Intraarterial chemotherapy for extremity osteosarcoma and MFH in adults. *Clin Orthop Relat Res.* 2008;466:1292–1301.
17. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1992;176:1693–1702.
18. Killion JJ, Fidler IJ. Systemic targeting of liposome-encapsulated immunomodulators to macrophages for treatment of cancer metastasis. *Immunomethods.* 1994;4:273–279.
19. Koido S, Hara E, Homma S, Torii A, Mitsunaga M, Yanagisawa S, Toyama Y, Kawahara H, Watanabe M, Yoshida S, Kobayashi S, Yanaga K, Fujise K, Tajiri H. Streptococcal preparation OK-432 promotes fusion efficiency and enhances induction of antigen-specific CTL by fusions of dendritic cells and colorectal cancer cells. *J Immunol.* 2007;178:613–622.
20. Kubista B, Trieb K, Blahovec H, Kotz R, Micksche M. Hyperthermia increases the susceptibility of chondro- and osteosarcoma cells to natural killer cell-mediated lysis. *Anticancer Res.* 2002;22:789–792.
21. Kumaratilake LM, Ferrante A. IL-4 inhibits macrophage-mediated killing of Plasmodium falciparum in vitro: a possible parasite-immune evasion mechanism. *J Immunol.* 1992;149:194–199.
22. Larsson SE, Lorentzon R, Boquist L. Immunotherapy with irradiated tumour cells and BCG in experimental osteosarcoma. *Acta Orthop Scand.* 1981;52:469–474.
23. Lee JW, Kim H, Kang HJ, Kim HS, Park SH, Kim IO, Ahn HS, Shin HY. Clinical characteristics and treatment results of pediatric osteosarcoma: the role of high dose chemotherapy with autologous stem cell transplantation. *Cancer Res Treat.* 2008;40: 172–177.
24. Leventhal BG. Immunotherapy of sarcomas. *Natl Cancer Inst Monogr.* 1981;56:183–187.
25. Liebau C, Roesel C, Schmidt S, Karreman C, Prisack JB, Bojar H, Merk H, Wolfram N, Baltzer AW. Immunotherapy by gene transfer with plasmids encoding IL-12/IL-18 is superior to IL-23/IL-18 gene transfer in a rat osteosarcoma model. *Anticancer Res.* 2004;24:2861–2867.
26. Lotze MT, Line BR, Mathisen DJ, Rosenberg SA. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): implications for the adoptive immunotherapy of tumors. *J Immunol.* 1980;125:1487–1493.
27. Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999;223:77–92.
28. Lutz MB, Rössner S. Factors influencing the generation of murine dendritic cells from bone marrow: the special role of fetal calf serum. *Immunobiology.* 2007;212:855–862.
29. Machak GN, Tkachev SI, Solovyev YN, Sinyukov PA, Ivanov SM, Kochergina NV, Ryjkov AD, Tepliakov VV, Bokhian BY, Glebovskaya VV. Neoadjuvant chemotherapy and local radiotherapy for high-grade osteosarcoma of the extremities. *Mayo Clin Proc.* 2003;78:147–155.
30. Meyers PA, Schwartz CL, Krailo M, Kleinerman ES, Betcher D, Bernstein ML, Conrad E, Ferguson W, Gebhardt M, Goorin AM, Harris MB, Healey J, Huvos A, Link M, Montebello J, Nadel H, Nieder M, Sato J, Siegal G, Weiner M, Wells R, Wold L, Womer R, Grier H. Osteosarcoma: a randomized, prospective trial of the addition of ifosfamide and/or muramyl tripeptide to cisplatin, doxorubicin, and high-dose methotrexate. *J Clin Oncol.* 2005; 23:2004–2011.
31. Monzavi-Karbassi B, Hennings LJ, Artaud C, Liu T, Jousheghany F, Pashov A, Murali R, Hutchins LF, Kieber-Emmons T. Preclinical studies of carbohydrate mimetic peptide vaccines for breast cancer and melanoma. *Vaccine.* 2007;25:3022–3031.
32. Morikawa Y, Tohya K, Ishida H, Matsuura N, Kakudo K. Different migration patterns of antigen-presenting cells correlate with Th1/Th2-type responses in mice. *Immunology.* 1995;85: 575–581.
33. Nakashima Y, Deie M, Yanada S, Sharman P, Ochi M. Magnetically labeled human natural killer cells, accumulated in vitro by an external magnetic force, are effective against HOS osteosarcoma cells. *Int J Oncol.* 2005;27:965–971.
34. Neff JR, Enneking WF. Adoptive immunotherapy in primary osteosarcoma: an interim report. *J Bone Joint Surg Am.* 1975; 57:145–148.
35. Nishida H, Tsuchiya H, Tomita K. Re-implantation of destructive tumour tissue treated by liquid nitrogen cryotreatment induces anti-tumour activity against murine osteosarcoma. *J Bone Joint Surg Br.* 2008;90:1249–1255.
36. Pelham JM, Gray JD, Flannery GR, Pimm MV, Baldwin RW. Interferon-alpha conjugation to human osteogenic sarcoma monoclonal antibody 791T/36. *Cancer Immunol Immunother.* 1983;15:210–216.
37. Research Promotion Bureau, Life Sciences Divisions. Fundamental guidelines for proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology. Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71. Available at: http://www.lifescience.mext.go.jp/policies/pdf/an_material011.pdf. Accessed October 29, 2007.
38. Romieu R, Baratin M, Kayibanda M, Guillet JG, Viguier M. IFN-gamma-secreting Th cells regulate both the frequency and avidity of epitope-specific CD8 + T lymphocytes induced by peptide immunization: an ex vivo analysis. *Int Immunol.* 1998;10:1273–1279.
39. Sabel MS, Kaufman CS, Whitworth P, Chang H, Stocks LH, Simmons R, Schultz M. Cryoablation of early-stage breast cancer: work-in-progress report of a multi-institutional trial. *Ann Surg Oncol.* 2004;11:542–549.
40. Schendel DJ, Gansbacher B, Oberneder R, Kriegmair M, Hofstetter A, Riethmüller G, Segurado OG. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines. *J Immunol.* 1993;151:4209–4220.
41. Slingluff CL Jr, Chianese-Bullock KA, Bullock TN, Grosh WW, Mullins DW, Nichols L, Olson W, Petroni G, Smolkin M, Engelhard VH. Immunity to melanoma antigens: from self-tolerance to immunotherapy. *Adv Immunol.* 2006;90:243–295.
42. Southam CM, Marcove R, Shanks E. Clinical trials of autogenous tumor vaccine for treatment of osteogenic sarcoma. *Proceedings of the Seventh National Cancer Conference.* Philadelphia, PA: JB Lippincott; 1973:91.

43. Tanzawa Y, Tsuchiya H, Yamamoto N, Sakayama K, Minato H, Tomita K. Histological examination of frozen autograft treated by liquid nitrogen removed 6 years after implantation. *J Orthop Sci.* 2008;13:259–264.
44. Terunuma H, Deng X, Dewan Z, Fujimoto S, Yamamoto N. Potential role of NK cells in the induction of immune responses: implications for NK cell-based immunotherapy for cancers and viral infections. *Int Rev Immunol.* 2008;27:93–110.
45. Toes RE, Blom RJ, Offringa R, Kast WM, Melief CJ. Enhanced tumor outgrowth after peptide vaccination: functional deletion of tumor-specific CTL induced by peptide vaccination can lead to the inability to reject tumors. *J Immunol.* 1996;156:3911–3918.
46. Tsuchiya H, Tomita K, Mori Y, Asada N, Morinaga T, Kitano S, Yamamoto N. Caffeine-assisted chemotherapy and minimized tumor excision for nonmetastatic osteosarcoma. *Anticancer Res.* 1998;18:657–666.
47. Tsuchiya H, Tomita K, Mori Y, Asada N, Yamamoto N. Marginal excision for osteosarcoma with caffeine assisted chemotherapy. *Clin Orthop Relat Res.* 1999;358:27–35.
48. Tsuchiya H, Wan SL, Sakayama K, Yamamoto N, Nishida H, Tomita K. Reconstruction using an autograft containing tumour treated by liquid nitrogen. *J Bone Joint Surg Br.* 2005;87:218–225.
49. Tsuchiya H, Yasutake H, Yokogawa A, Baba H, Ueda Y, Tomita K. Effect of chemotherapy combined with caffeine for osteosarcoma. *J Cancer Res Clin Oncol.* 1992;118:567–569.
50. Urano M, Tanaka C, Sugiyama Y, Miya K, Saji S. Antitumor effects of residual tumor after cryoablation: the combined effect of residual tumor and a protein-bound polysaccharide on multiple liver metastases in a murine model. *Cryobiology.* 2003;46:238–245.
51. Visonneau S, Cesano A, Jeglum KA, Santoli D. Adjuvant treatment of canine osteosarcoma with the human cytotoxic T-cell line TALL-104. *Clin Cancer Res.* 1999;5:1868–1875.
52. Warren RQ, Tsang KY. Induction of immunity to a human osteosarcoma-associated antigen in mice using anti-idiotypic antibodies. *Clin Immunol Immunopathol.* 1990;56:334–343.
53. Yamamoto N, Tsuchiya H, Tomita K. Effects of liquid nitrogen treatment on the proliferation of osteosarcoma and the biomechanical properties of normal bone. *J Orthop Sci.* 2003;8:374–380.

JCM
Journals.ASM.org

NS5A Sequence Heterogeneity of Hepatitis C Virus Genotype 4a Predicts Clinical Outcome of Pegylated-Interferon–Ribavirin Therapy in Egyptian Patients

Ahmed El-Shamy,[a,b]* Ikuo Shoji,[a] Wafaa El-Akel,[c] Shymaa E. Bilasy,[d] Lin Deng,[a] Maissa El-Raziky,[c] Da-peng Jiang,[a] Gamal Esmat,[c] and Hak Hotta[a]

Division of Microbiology, Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Japan[a]; Department of Virology, Suez Canal University Faculty of Veterinary Medicine, Ismalia, Egypt[b]; Department of Tropical Medicine, Cairo University, Cairo, Egypt[c]; and Department of Biochemistry, Suez Canal University Faculty of Pharmacy, Ismalia, Egypt[d]

Hepatitis C virus genotype 4 (HCV-4) is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world. HCV-4 infection is common in the Middle East and Africa, with an extraordinarily high prevalence in Egypt. Viral genetic polymorphisms, especially within core and NS5A regions, have been implicated in influencing the response to pegylated-interferon and ribavirin (PEG-IFN/RBV) combination therapy in HCV-1 infection. However, this has not been confirmed in HCV-4 infection. Here, we investigated the impact of heterogeneity of NS5A and core proteins of HCV-4, mostly subtype HCV-4a, on the clinical outcomes of 43 Egyptian patients treated with PEG-IFN/RBV. Sliding window analysis over the carboxy terminus of NS5A protein identified the IFN/RBV resistance-determining region (IRRDR) as the most prominent region associated with sustained virological response (SVR). Indeed, 21 (84%) of 25 patients with SVR, but only 5 (28%) of 18 patients with non-SVR, were infected with HCV having IRRDR with 4 or more mutations (IRRDR ≥ 4) ($P = 0.0004$). Multivariate analysis identified IRRDR ≥ 4 as an independent SVR predictor. The positive predictive value of IRRDR ≥ 4 for SVR was 81% (21/26; $P = 0.002$), while its negative predictive value for non-SVR was 76% (13/17; $P = 0.02$). On the other hand, there was no significant correlation between core protein polymorphisms, either at residue 70 or at residue 91, and treatment outcome. In conclusion, the present results demonstrate for the first time that IRRDR ≥ 4, a viral genetic heterogeneity, would be a useful predictive marker for SVR in HCV-4 infection when treated with PEG-IFN/RBV.

Hepatitis C virus (HCV) is a major cause of chronic liver disease, hepatocellular carcinoma, and deaths from liver disease and is the most common indication for liver transplantation (7, 26–28, 38). HCV has been classified into seven major genotypes and a series of subtypes (35, 36). In general, HCV genotype 4 (HCV-4) is common in the Middle East and Africa, where it is responsible for more than 80% of HCV infections (23). Although HCV-4 is the cause of approximately 20% of the 180 million cases of chronic hepatitis C in the world, it has not been a major subject of research.

Egypt has the highest prevalence of HCV worldwide (15%) and the highest prevalence of HCV-4, which is responsible for 90% of the total HCV infections, with a predominance of the subtype 4a (HCV-4a) (1, 32). This extraordinarily high prevalence results in an increasing incidence of hepatocellular carcinoma in Egypt, which is now the second most frequent cause of cancer and cancer mortality among men (17, 21). More than 2 decades have passed since the discovery of HCV, and yet therapeutic options remain limited. Up to 2011, the standard treatment for chronic hepatitis C consisted of pegylated alpha interferon (PEG-IFN) and ribavirin (RBV) (19); however, by May 2011 two protease inhibitors (telaprevir and boceprevir) were approved by the Food and Drug Administration (FDA) for use in combination with PEG-IFN/RBV for adult chronic hepatitis C patients with HCV genotype 1 (24, 34). Since the approval of these new protease inhibitors for treatment of HCV-1 infection, the response of HCV-4 to the standard regimen of treatment (PEG-IFN/RBV) has lagged behind other genotypes and HCV-4 has become the most resistant genotype to treat. As PEG-IFN/RBV still remains to be used to treat HCV-4-infected patients, exploring the factors that predict the outcome of PEG-IFN/RBV treatment, such as sustained virological response (SVR), for HCV-4 infections is needed to assess more accurately the likelihood of SVR and thus to make more informed treatment decisions.

While the SVR rate for PEG-IFN/RBV treatment hovers at 50 to 60% in HCV-1 and -4 infection, it is up to 80% in HCV-2 and -3 infections (19, 33). This difference in responses among patients infected with different HCV genotypes suggests that viral genetic heterogeneity could affect, at least to some extent, the sensitivity to IFN-based therapy. In this context, the correlation between IFN-based therapy outcome and sequence polymorphisms within the viral core and NS5A proteins has been widely discussed, in particular in regard to Japanese patients with HCV-1b infection. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A of HCV-1b, called the IFN sensitivity-determining region (ISDR), were correlated with IFN responsiveness (18). Subsequently, in the era of PEG-IFN/

Received 8 August 2012 **Returned for modification** 30 August 2012
Accepted 14 September 2012
Published ahead of print 19 September 2012
Address correspondence to Hak Hotta, hotta@kobe-u.ac.jp.
* Present address: Ahmed El-Shamy, Division of Liver Diseases, Mount Sinai School of Medicine, New York, New York, USA.

doi:10.1128/JCM.02109-12

RBV combination therapy, we identified a new region near the C terminus of NS5A, referred to as the IFN/RBV resistance-determining region (IRRDR) (13). Recently, we also demonstrated the correlation between IRRDR polymorphism and PEG-IFN/RBV treatment outcome in HCV-2a and -2b infections (15). In addition, HCV core protein polymorphism, in particular at positions 70 and 91, was also proposed as a pretreatment predictor of poor virological response in patients infected with HCV-1b (4–6). To the best of our knowledge, there is no information regarding the correlation between sequence heterogeneity in the NS5A and core proteins of HCV-4 and PEG-IFN/RBV treatment outcome. In the present study, we aimed to investigate this issue in Egyptian patients infected with HCV-4.

MATERIALS AND METHODS

Ethics statement. The study protocol, which conforms to the provisions of the Declaration of Helsinki, was approved beforehand by the Ethic Committees in Cairo University Hospital and in Kobe University, and written informed consent was obtained from each patient prior to the treatment.

Patients. A total of 43 previously untreated patients who were chronically infected with HCV-4a (34 patients), HCV-4m (3 patients), HCV-4n (3 patients), or HCV-4o (3 patients) were consecutively evaluated for antiviral treatment at Cairo University Hospital, Cairo, Egypt, between January 2008 and September 2010. The HCV subtype was determined according to the method of Okamoto et al. (31). The patients were treated with PEG-IFN α-2a (180 μg/week, subcutaneously) and RBV (1,000 to 1,200 mg daily, *per os*) for 48 weeks. The quantification of serum HCV RNA titers was performed as previously reported (14). To minimize the therapeutic burdens, including the high cost and possible side effects, therapy was discontinued if HCV RNA titers at week 12 did not drop by 2 log compared with baseline values or if HCV RNA was still detectable at week 24. These were considered a null response (see Results).

Sequence analysis of the NS5A and core regions of the HCV genome. Blood samples were collected using Vacutainer tubes. The sera were separated within 2 h of blood collection, transferred to sterile cryovials, and kept frozen at −80°C until use. HCV RNA was extracted from 140 μl of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). The extracted RNA was reverse transcribed and amplified for the HCV genome encoding a carboxy terminus of NS5A (amino acids [aa] 2193 to 2417) and the core protein (aa 1 to 191) using SuperScript III one-step RT-PCR Platinum *Taq* HiFi (Invitrogen, Tokyo, Japan). The resultant reverse transcription (RT)-PCR product was subjected to a second-round PCR by using Platinum *Taq* DNA polymerase high fidelity III (Invitrogen). Primers used for amplification of the 3′ half of the NS5A region of HCV-4 were as follows: NS5A-4/F1 (5′-CTCAAYTCGTTCGT RGTGGGATC-3′; sense) and NS5A-4/R1 (5′-CGAAGGTCACCTTCTT CTGCCG-3′; antisense) for one-step RT-PCR; and NS5A-4/F2 (5′-ATG CGAGCCYGAGCCGGACGT-3′; sense) and NS5A-4/R2 (5′-GCTCAGG GGGYTRATTGGCAGCT-3′; antisense) for the second-round PCR. Primers for amplification of the core region of HCV-4 were 249-F (5′-G CTAGCCGAGTAGTGTTG-3′; sense) and 984-R (5′-GATGTGRTGRTC GGCCTC-3′; antisense) (40) for one-step RT-PCR; and 319-F (5′-GGA GGTCTCGTAGACCGTGC-3′; sense) (40) and primer-186 (5′-ATGTA CCCCATGAGGTCGGC-3′; antisense) (2) for the second-round PCR. RT was performed at 45°C for 30 min and terminated at 94°C for 2 min, followed by the first-round PCR over 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 68°C for 90 s. The second-round PCR was performed under the same conditions. The sequences of the amplified fragments were determined by direct sequencing without subcloning. The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan). The numbering of amino acid residues for HCV-4 isolates is according to the polyprotein of ED43 isolate (accession no. Y11604) (10). Consensus sequences of the carboxy terminus of NS5A of a given HCV-4 subtype were inferred by alignment of all sequences obtained in this study as well as all available NS5A sequences of HCV-4a (accession no. Y11604, DQ418782 to DQ418789, DQ516084, and DQ988073 to DQ988079), HCV-4m (FJ462433), HCV-4n (FJ462441), and HCV-4o (FJ462440) from the databases.

Statistical analysis. Numerical data were analyzed by Student's *t* test and categorical data by Fisher's exact probability test. To evaluate the optimal threshold of the number of amino acid mutations in IRRDR for prediction of treatment outcomes, the receiver operating characteristic (ROC) curve was constructed. Univariate and multivariate logistic regression analyses were performed to identify independent predictors for treatment outcomes. All statistical analyses were performed using the SPSS version 16 software (SPSS Inc., Chicago, IL). Unless otherwise stated, a P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB725987 through AB726066.

TABLE 1 Virological responses of HCV-4-infected patients treated with PEG-IFN/RBV

Virological response	Proportion (%) of patients with indicated response (no. of patients/total no.)				
	HCV-4[a]	HCV-4a	HCV-4 m	HCV-4n	HCV-4o
SVR	58 (25/43)	56 (19/34)	100 (3/3)	33 (1/3)	67 (2/3)
Non-SVR	42 (18/43)	44 (15/34)	0 (0/3)	67 (2/3)	33 (1/3)
Null response	30 (13/43)	32 (11/34)	0 (0/3)	67 (2/3)	0 (0/3)
Relapse	12 (5/43)	12 (4/34)	0 (0/3)	0 (0/3)	33 (1/3)

[a] Includes all 43 cases with HCV-4 infection (34 cases with HCV-4a and 3 cases each with HCV-4m, -4n, and -4o).

RESULTS

Patients' responses to PEG-IFN/RBV combination therapy. Among 43 patients enrolled in this study, 30 (70%) patients completed the entire course of PEG-IFN/RBV treatment for 48 weeks and follow-up for 24 weeks. On the other hand, the treatment was discontinued for 13 (30%) patients due to poor virological responses at 12 or 24 weeks after initiation of the therapy. Overall, 25 (58%) patients achieved SVR while 18 (42%) patients had non-SVR (Table 1). When analyzed on the basis of the subtype classification, SVR was achieved by 56% (19/34), 100% (3/3), 33% (1/3), and 67% (2/3) of patients infected with HCV-4a, -4m, -4n, and -4o, respectively.

Non-SVR patients are classified into two groups: (i) patients with null response, who did not achieve >2-log reduction of the initial viral load at week 12 or who had detectable viremia at week 24 of the treatment period; and (ii) patients with relapse, who were negative for HCV-RNA at the end of the treatment period (week 48) followed by a rebound viremia at a certain time point during the follow-up period of 24 weeks. Patients with null response represented 30% (13/43) of all the HCV-4-infected subjects analyzed, while those with relapse represented 12% (5/43). A similar tendency was observed for subtype HCV-4a.

Among various patients' demographic characteristics, SVR patients had a significantly lower average age than that of non-SVR patients (Table 2). Furthermore, a tendency for SVR patients to have a lower average titer of initial viral load than that of non-SVR was noted, although the difference was not statistically significant, due possibly to the small number of patients analyzed ($P = 0.07$).

TABLE 2 Demographic characteristics of HCV-4-infected patients with SVR and non-SVR[a]

Factor	SVR	Non-SVR	*P* value
Age	38.47 ± 9.51	45.80 ± 5.65	0.014
Sex (male/female)	18/7	15/3	0.48
BMI	27.36 ± 3.65	27.67 ± 5.28	0.85
Platelets ($\times 10^3/\mu l$)	204.4 ± 40.63	216.7 ± 87.25	0.59
Hemoglobin (g/dl)	14.54 ± 1.38	15.08 ± 1.39	0.25
WBC count	7,041 ± 1,876	7,078 ± 2,977	0.96
Albumin (g/dl)	4.12 ± 0.36	4.328 ± 0.41	0.11
ALT (IU/liter)	78.72 ± 59.68	82.39 ± 41.80	0.83
AST (IU/liter)	64.94 ± 27.63	58.17 ± 23.98	0.44
HCV-RNA (IU/ml)	84,290 ± 186,300	501,800 ± 816,700	0.07

[a] Values are means ± standard deviations. SVR, sustained virological response; BMI, body mass index; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Correlation between NS5A sequence heterogeneity and SVR in HCV-4 infection. We and other researchers reported significant correlation between sequence polymorphisms within the C-terminal half of NS5A, including that in ISDR and IRRDR, and PEG-IFN/RBV treatment outcome in HCV-1 and HCV-2 infections (13, 15, 18, 30). However, this information is quite limited in HCV-4 infection. To clarify this issue, part of the HCV-4 genome encoding a carboxy terminus (aa 2193 to 2417) of NS5A in pretreatment sera was amplified and sequenced, and amino acid sequences were deduced. The sequences obtained as well as all available NS5A sequences of HCV-4a, -4m, -4n, and -4o from the databases were aligned, and the consensus sequences for a desired HCV-4 subtype were inferred (see Materials and Methods). Next, to identify an NS5A region(s) that would be significantly correlated with treatment outcome, we carried out a sliding window analysis with a window size of 30 residues over the C-terminal half (aa 2193 to 2417) of NS5A sequences obtained from all SVR (n = 25) and non-SVR (n = 18) patients along with corresponding consensus sequences of each HCV-4 subtype as described previously (30). This analysis revealed that the difference in the overall number of amino acid mutations between SVR and non-SVR isolates exceeded the significant threshold only in a region corresponding to IRRDR of HCV-1b (13), ranging from aa 2331 to 2383, thus being referred to as IRRDR[HCV-4] (Fig. 1). Indeed, the average number of amino acid mutations in IRRDR[HCV-4] was significantly larger in SVR than in non-SVR (P = 0.0005) isolates (Fig. 2A). Sequences of IRRDR of HCV-4a, -4m, -4n, and -4o obtained from SVR and non-SVR patients along with the number of IRRDR mutations of each isolate are shown in Fig. 2B.

Next, we performed ROC curve analysis to estimate the optimal cutoff number of IRRDR[HCV-4] mutations for SVR prediction. This analysis estimated 4 mutations as the optimal number of IRRDR[HCV-4] mutations to predict SVR, since it achieved the highest sensitivity (84%; sensitivity refers to the proportion of SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 4 or more mutations) and specificity (72%; specificity refers to the proportion of non-SVR patients who were infected with HCV isolates of IRRDR[HCV-4] with 3 or fewer mutations) with an area under the curve (AUC) of 0.82 (Fig. 3). Accordingly, 21 (84%) of 25 patients with SVR, in contrast to only 5 (28%) of 18 patients with non-SVR, had IRRDR[HCV-4] with 4 or more mutations (referred to as IRRDR[HCV-4]≥4), with the difference between the two groups being statistically significant (P = 0.0004) (Table 3). It should be noted that 4 (31%) of 13 patients with null response and only 1 (20%) of 5 patients with relapse had HCV with IRRDR[HCV-4]≥4. These results collectively suggest that IRRDR[HCV-4]≥4 is significantly associated with SVR. In this connection, we also tested the impact of a higher (≥5) and a lower (≥3) degree of IRRDR mutations on treatment outcome. IRRDR[HCV-4]≥5 was significantly associated with SVR, though with a relatively lower sensitivity (64%) than that of IRRDR[HCV-4]≥4 (Table 3). On the other hand, there was no significant correlation between IRRDR[HCV-4]≥3 and SVR.

Correlation between core protein sequence heterogeneity and SVR in HCV-4 infection. A close correlation between core protein sequence patterns at positions 70 and 91 and treatment outcome has been proposed, especially in Japanese patients with HCV-1b infection (4–6). To examine this hypothesis in Egyptian patients infected with HCV-4, core sequences of the viral genome were amplified from the pretreated sera, and the amino acid sequences were deduced. Due to a high degree of sequence homology among core sequences of various HCV-4 subtypes, all sequences obtained were aligned with the prototype sequence, ED43 (10). The residues at positions 70 and 91 were both well conserved among the sequences analyzed, and therefore, no correlation with treatment outcome was observed for these residues (Fig. 4). All but two isolates had arginine at position 70 (Arg^{70}), the residue that has been associated with an IFN-sensitive phenotype as far as the core protein of HCV-1b is concerned (4–6). On the other hand, Pro at position 71 showed a tendency to be more frequent in SVR than in non-SVR patients; however, the frequency was not statistically different between the two groups.

Identification of independent predictive factors for SVR in HCV-4 infection. In order to identify significant independent

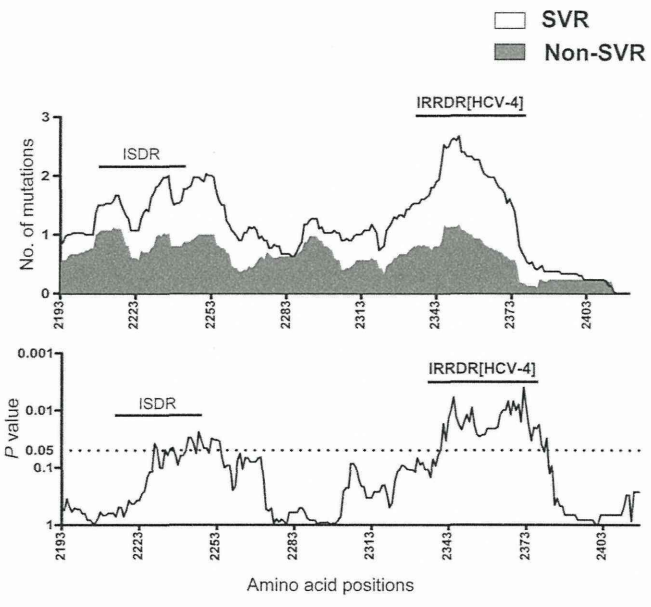


FIG 1 Sliding window analysis over the carboxy terminus (aa 2193 to 2417) of NS5A of HCV-4 obtained from SVR and non-SVR patients.

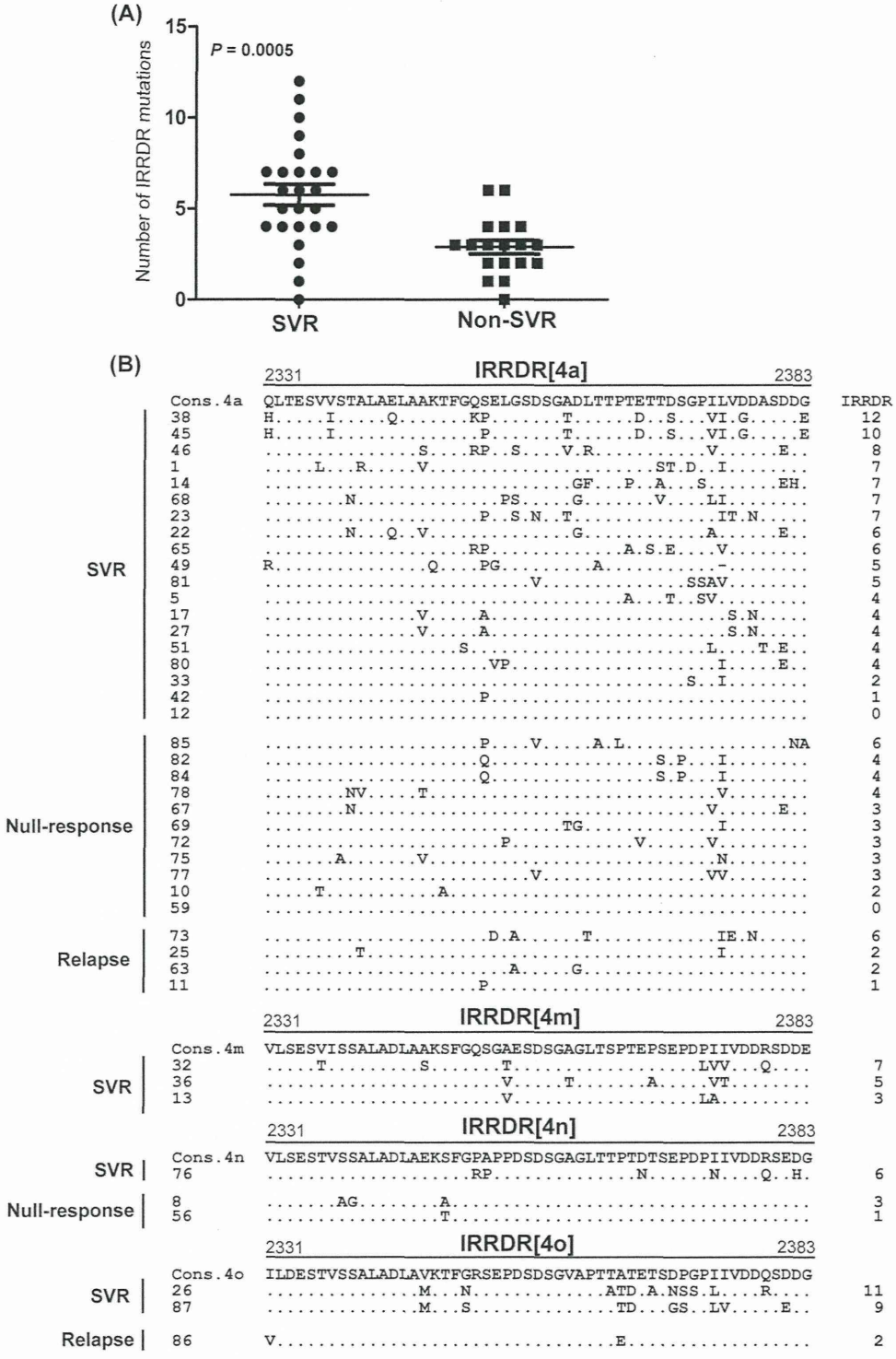


FIG 2 Correlation between IRRDR[HCV-4] sequence variations and treatment outcome. (A) Average number of amino acid mutations in IRRDR[HCV-4] obtained from SVR and non-SVR patients. (B) Alignment of IRRDR[HCV-4] sequences obtained from SVR and non-SVR patients with HCV-4a, -4m, -4n, and -4o. The consensus sequence (Cons) of each subtype is shown on the top. The numbers along the sequence indicate the amino acid positions. Dots indicate residues identical to those of the Cons sequence. The numbers of the mutations in each IRRDR (4a, 4m, 4n, or 4o) are shown on the right.

predictive factors of SVR for PEG-IFN/RBV treatment outcome in HCV-4 infection, first, all available data of baseline patients' parameters and IRRDR[HCV-4] polymorphism were entered in a univariate logistic analysis. This analysis yielded 3 factors that were correlated or nearly correlated with SVR: IRRDR[HCV-4]≥4 ($P = 0.0004$), patient's age (<42 years; $P = 0.03$), and HCV RNA titer (<5,200 IU/ml; $P = 0.08$). Subsequently, these 3 factors were entered in multivariate logistic regression analysis. This anal-

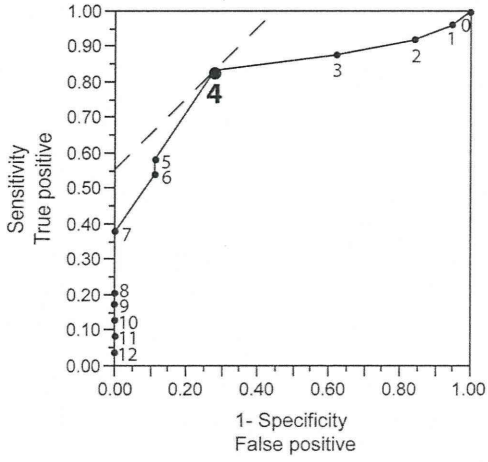


FIG 3 ROC curve analysis of IRRDR[HCV-4] sequence heterogeneity for SVR prediction. The solid line curve shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR mutations analyzed. The dashed line in the upper left corner indicates the optimal number of IRRDR[HCV-4] mutations for SVR prediction, which yields the highest sensitivity (84%) and the highest specificity (72%).

ysis revealed that the IRRDR[HCV-4]≥4 was the only independent predictive factor for SVR in HCV-4 infection (Table 4). We then assessed SVR predictability by means of IRRDR[HCV-4]≥4. As shown in Table 5, IRRDR[HCV-4]≥4 would predict SVR with a positive predictive value (PPV) of 81% ($P = 0.002$) and sensitivity of 84%. On the other hand, IRRDR[HCV-4]≤3 would predict non-SVR with a negative predictive value (NPV) of 76% ($P = 0.02$) and specificity of 72%. Thus, the degree of sequence variation in IRRDR[HCV-4] would yield useful positive and negative predictive markers for PEG-IFN/RBV therapy outcome in HCV-4-infected patients.

DISCUSSION

Both host and viral genetic factors have been implicated in influencing the clinical response to PEG-IFN/RBV therapy for HCV infection (22). It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a critical impact on the treatment outcome of patients infected with HCV-1 (20, 37, 39). As for the viral factor(s), polymorphisms of NS5A and core regions of a given HCV genotype have been linked to a difference in SVR rates (3, 4, 13, 18, 30). This hypothesis was mostly inferred from studies carried out with Asian populations, in particular Japanese, with HCV-1b infection. However, whether it can be applied to non-Asian populations infected with non-HCV-1 is still unknown. To the best of our knowledge, this is the first study that specifically examines the relationship between HCV genome heterogeneity, in particular in NS5A and core regions, and PEG-IFN/RBV treatment outcome in Egyptian patients infected with HCV-4. In analogy with our previous studies that identified IRRDR as a significant determinant for PEG-IFN/RBV treatment outcome in Japanese patients infected with HCV-1b, -2a, and -2b (12–16), we have demonstrated in the present study that sequence heterogeneity within IRRDR is closely associated with the ultimate treatment outcome in Egyptian patients infected with HCV-4. A high degree of sequence variation in IRRDR[HCV-4], i.e., more than 4 (IRRDR ≥ 4), significantly correlated with SVR, while a low degree of sequence variation in this region (IRRDR ≤ 3) correlated with non-SVR, null response, and relapse. The majority of patients with SVR (84%) had HCV with IRRDR of ≥4. In contrast, nearly two-thirds (72%) of the patients with non-SVR had HCV with IRRDR ≤ 3 ($P = 0.0004$) (Table 3). Notably, 21 of the 26 patients infected with HCV with IRRDR[HCV-4]≥4 achieved SVR. Accordingly, the PPV and NPV of IRRDR[HCV-4]≥4 for SVR and non-SVR patients were 81% ($P = 0.002$) and 76% ($P = 0.02$), respectively (Table 5). Our present results thus strongly suggest that the degree of sequence heterogeneity within IRRDR[HCV-4] would be a useful marker for prediction of treatment outcome in HCV-4 infection.

The molecular mechanism underlying the possible involvement of this region in IFN responsiveness of the virus is still unknown. The significant difference among IRRDR sequence patterns may suggest genetic flexibility of this region. Indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions (29). This flexibility might play an important role in modulating the interaction with various host systems, including IFN-induced antiviral machineries. It is also possible that the genetic flexibility of IRRDR is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN-based therapy (8, 29, 41). Also, it is worth noting that IRRDR is among the most variable sequences across the different genotypes and subtypes of HCV (25) whereas its upstream and downstream sequences show a higher degree of sequence conservation (15). This may suggest that whereas the upstream and downstream sequences have a conserved function(s) across all the HCV genotypes, IRRDR sequences have a genotype-dependent or even a strain-dependent function(s).

A mutation at position 70 of the core protein of HCV-1b has been reported to be correlated with PEG-IFN/RBV treatment out-

TABLE 3 Correlation between NS5A sequence heterogeneity and virological responses in HCV-4 infection

Factor	No. of isolates/total no. (%)				*P* value for SVR versus:		
	SVR	Non-SVR	Null response	Relapse	Non-SVR	Null response	Relapse
IRRDR ≥ 4	21/25 (84)[a]	5/18 (28)	4/13 (31)	1/5 (20)	0.0004	0.003	0.01
IRRDR ≤ 3	4/25 (16)	13/18 (72)[b]	9/13 (69)	4/5 (80)			
IRRDR ≥ 5	16/25 (64)[a]	2/18 (11)	1/13 (8)	1/5 (20)	0.0006	0.002	0.14
IRRDR ≤ 4	9/25 (36)	16/18 (89)[b]	12/13 (92)	4/5 (80)			
IRRDR ≥ 3	22/25 (88)[a]	11/18 (61)	10/13 (77)	1/5 (20)	0.066	0.39	0.006
IRRDR ≤ 2	3/25 (12)	7/18 (39)[b]	3/13 (23)	4/5 (80)			

[a] Sensitivity (proportion of SVR patients with the favorable factor).
[b] Specificity (proportion of non-SVR patients with the unfavorable factor).