TABLE III. Baseline Characteristics of the Patients With or Without Lymphoproliferative Disorders

Features[a]	Lymphoproliferative disorders		Differences P value
	With (n = 57)	Without (n = 17)	
Age (years)	58.3 ± 14.5	55.2 ± 12.4	0.430
Men	35 (61%)	11 (65%)	0.805
Cirrhosis	15 (28%)	2 (12%)	0.177
Hepatocellular carcinoma	12 (22%)	2 (12%)	0.345
ALT (IU/L [5–25][b])	80 ± 82	170 ± 232	0.025
Platelets ($\times 10^4$/mm^3 [15–40][b])	17.6 ± 6.5	16.2 ± 6.4	0.430
HCV serogroup 1	47 (82%)	11 (65%)	0.119
HCV RNA in serum (log/ml)[c]	7.0 ± 1.4	6.8 ± 1.7	0.730
Serum HCV RNA > 5.0 log/ml	50 (88%)	14 (82%)	0.570
HCV RNA in B cells[d]	41 (72%)	6 (35%)	0.006
HCV RNA in B cells (log copies)[c]	4.5 ± 3.2	2.5 ± 3.4	0.036

ALT, alanine aminotranspherase; NS, not significant.
[a]Data are no (%) or the mean ± SD.
[b]Normal interquartile range.
[c]Determined in 100 ng of RNA extracted from cells.
[d]Determined number and percentage of patients with HCV RNA positive in B cells.

HCV infection. Either or both of the association of HCV-E2 protein with CD81 and the infection of B cells with HCV are proposed to accelerate the clonality of B cells [Matsuura et al., 2001].

In the 75 patients with chronic hepatitis C, the frequency of B cells harboring HCV RNA, as well as HCV RNA titers in B cells, was 10-fold higher than those of the other lymphoid cells including $CD4^+$, $CD8^+$ T cells. The replication of HCV in B cells was demonstrated in some patients with high titers of serum HCV RNA by the detection of negative-strand HCV RNA species; they represent viral replication intermediates. Combined, these results strongly suggest that HCV has a tropism for B cells.

On the basis of B-cell tropism, HCV isolates might be classified into at least three subgroups. One subgroup is merely associated with the surface receptors of B cells, but does not replicate efficiently in these cells. The results indicate that most HCV isolates belong to this group. Such an association might induce signaling toward a prolonged cell survival. B cells might express unknown receptors for HCV at levels higher than the other lymphoid cells. In support of this view, the negative-strand HCV RNA is barely detected in PBMCs from patients with hepatitis C, although positive strands are found in these cells [Lanford et al., 1995]. CD81, which is proposed as one of HCV receptors, is expressed on B cells much more densely than on hepatocytes [Machida et al., 2005]. There would be another subgroup of HCV capable of infecting B cells and replicating efficiently in them. Such B-cell tropic HCV, however, was identified in only four (5%) patients in this study. Nonetheless, infection with HCV may trigger somatic mutations in B cells, for example, bcl-6, p53, and β-catenin, leading to their clonal expansion [Machida et al., 2004]. A third subgroup of HCV would neither infect nor adhere to B cells.

It needs to be pointed out that methods used to detect HCV infection in extrahepatic cells have not combined high levels of both sensitivity and specificity, so far. Therefore, the possibility remains for the replication of HCV in some patients with chronic hepatitis C who did not have negative-strand HCV RNA in B cells in the present study; the frequency of false-negative results could not be determined in them. More sensitive and specific assay systems are required for estimating the actual frequency of HCV replication in B cells in patients with chronic hepatitis C with or without non-Hodgkin's lymphoma.

The association of HCV was less frequent in T cells than in the cell fraction without markers for B or T in the present study. The non-B, non-T cell fraction contains dendritic cells, macrophages and other lymphoid cells that were not $CD4^+$, $CD8^+$, or $CD19^+$. Dendritic cells have been demonstrated to interact with HCV-like particles in vitro [Barth et al., 2005], and are infected with HCV in vivo [Kanto et al., 2004]. Radkowski et al. [2005] reported the persistence of HCV in macrophages, even after it has been eliminated by interferon therapy. It is possible that HCV RNA might be associated with or infect dendritic cells and/or macrophages in non-B, non-T cell fractions, in replication levels lower than those in B cells.

The correlation between HCV infection and cryoglobulinemia is established [Agnello et al., 1992; Agnello, 1995]. RF was detected in high levels in sera from patients with not only chronic hepatitis C but also chronic hepatitis B (Table II). Recently, it was reported that the patients with chronic HBV infection are nearly three-times more likely to develop non-Hodgkin's lymphoma than controls [Ulcickas Yood et al., 2007]. As HCV infection, therefore, HBV infection may lead to lymphoproliferative disorders. The frequency of low CH_{50} levels was higher, although low C4 levels were detected only 2% in patients with chronic hepatitis C (Table II). These results stand at variance with those in a French study [Dumestre-Perard et al., 2002], which has shown low levels of both C4 and CH_{50} among patients infected with HCV. In this study, no patients with chronic hepatitis C had any cryoglobulinemia-related clinical syndrome, such as skin rashes, membranoproliferative glomerulonephritis and neuritis.

Hence, low C4, rather than CH_{50}, levels might be pathogenic and induce immune reactions in patients with chronic hepatitis C.

A correlation was sought for between infection and/or association of B cells with HCV and the occurrence of lymphoproliferative disorders. HCV RNA in B cells was an independent factor correlated with at least one of markers for lymphoproliferative disorders in multivariate analysis. Therefore, infection and/or association of B cells with HCV may lead to lymphoproliferative disorders, although the mechanism remains unknown. It is possible that infection of B cells with HCV would induce somatic mutations or over-expression of anti-apoptotic genes toward a prolonged survival of activated B cells. Or else, mere interaction between envelope proteins of HCV and signaling receptors on the cell surface, which regulate the survival of B cells, can be involved in the genesis of lymphoproliferative disorders.

The clonal expansion of B cells was reported to occur in 26% of Italian patients [Pozzato et al., 1999], while it has not been observed in any Japanese patient investigated so far. The detection of B-cell clonality in 11% of Japanese patients in this study, however, would point to a possibility for HCV-induced lymphoproliferation not dependent on ethnicity. Several studies have focused on important roles of sustained antigenic stimulation, analogous to lymphomagenesis due to infection with *H. pylori*, in a possible relevance to the extra-nodal marginal-zone B-cell lymphoma arising in lymphoid tissues on mucosae (MALT lymphoma) [Ivanovski et al., 1998; De Re et al., 2000; Sansonno et al., 2004]. Further studies are necessary to clarify molecular mechanisms for the generation of lymphoproliferative disorders and the correlation between malignant lymphoma and lymphoproliferative disorders.

REFERENCES

Agnello V. 1995. The aetiology of mixed cryoglobulinaemia associated with hepatitis C virus infection. Scand J Immunol 42:179–184.

Agnello V, Chung RT, Kaplan LM. 1992. A role for hepatitis C virus infection in type II cryoglobulinemia. N Engl J Med 327:1490–1495.

Barth H, Ulsenheimer A, Pape GR, Diepolder HM, Hoffmann M, Neumann-Haefelin C, Thimme R, Henneke P, Klein R, Paranhos-Baccala G, Depla E, Liang TJ, Blum HE, Baumert TF. 2005. Uptake and presentation of hepatitis C virus-like particles by human dendritic cells. Blood 105:3605–3614.

Cacoub P, Poynard T, Ghillani P, Charlotte F, Olivi M, Piette JC, Opolon P. 1999. Extrahepatic manifestations of chronic hepatitis C. MULTIVIRC Group. Multidepartment Virus C. Arthritis Rheum 42:2204–2212.

Castillo I, Rodriguez-Inigo E, Bartolome J, de Lucas S, Ortiz-Movilla N, Lopez-Alcorocho JM, Pardo M, Carreno V. 2005. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. Gut 54:682–685.

De Re V, De Vita S, Marzotto A, Rupolo M, Gloghini A, Pivetta B, Gasparotto D, Carbone A, Boiocchi M. 2000. Sequence analysis of the immunoglobulin antigen receptor of hepatitis C virus-associated non-Hodgkin lymphomas suggests that the malignant cells are derived from the rheumatoid factor-producing cells that occur mainly in type II cryoglobulinemia. Blood 96:3578–3584.

Donada C, Crucitti A, Donadon V, Tommasi L, Zanette G, Crovatto M, Santini GF, Chemello L, Alberti A. 1998. Systemic manifestations and liver disease in patients with chronic hepatitis C and type II or III mixed cryoglobulinaemia. J Viral Hepat 5:179–185.

Dumestre-Perard C, Ponard D, Drouet C, Leroy V, Zarski JP, Dutertre N, Colomb MG. 2002. Complement C4 monitoring in the follow-up of chronic hepatitis C treatment. Clin Exp Immunol 127:131–136.

Ferri C, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, Lombardini F, Greco F, Capochiani E, Mazzoni A, Mazzaro C, Pasero G. 1994. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. Br J Haematol 88:392–394.

Frangeul L, Musset L, Cresta P, Cacoub P, Huraux JM, Lunel F. 1996. Hepatitis C virus genotypes and subtypes in patients with hepatitis C, with and without cryoglobulinemia. J Hepatol 25:427–432.

Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, Arase Y, Fukuda M, Chayama K, Murashima N, Kumada H. 1998. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: A prospective observation of 2215 patients. J Hepatol 28:930–938.

Ito T, Yasui K, Mukaigawa J, Katsume A, Kohara M, Mitamura K. 2001. Acquisition of susceptibility to hepatitis C virus replication in HepG2 cells by fusion with primary human hepatocytes: Establishment of a quantitative assay for hepatitis C virus infectivity in a cell culture system. Hepatology 34:566–572.

Ivanovski M, Silvestri F, Pozzato G, Anand S, Mazzaro C, Burrone OR, Efremov DG. 1998. Somatic hypermutation, clonal diversity, and preferential expression of the VH 51p1/VL kv325 immunoglobulin gene combination in hepatitis C virus-associated immunocytomas. Blood 91:2433–2442.

Kanto T, Inoue M, Miyatake H, Sato A, Sakakibara M, Yakushijin T, Oki C, Itose I, Hiramatsu N, Takehara T, Kasahara A, Hayashi N. 2004. Reduced numbers and impaired ability of myeloid and plasmacytoid dendritic cells to polarize T helper cells in chronic hepatitis C virus infection. J Infect Dis 190:1919–1926.

Lanford RE, Chavez D, Chisari FV, Sureau C. 1995. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 69:8079–8083.

Laskus T, Radkowski M, Piasek A, Nowicki M, Horban A, Cianciara J, Rakela J. 2000. Hepatitis C virus in lymphoid cells of patients coinfected with human immunodeficiency virus type 1: Evidence of active replication in monocytes/macrophages and lymphocytes. J Infect Dis 181:442–448.

Lerat H, Rumin S, Habersetzer F, Berby F, Trabaud MA, Trepo C, Inchauspe G. 1998. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: Influence of viral load, viral genotype, and cell phenotype. Blood 91:3841–3849.

Machida K, Cheng KT, Sung VM, Shimodaira S, Lindsay KL, Levine AM, Lai MY, Lai MM. 2004. Hepatitis C virus induces a mutator phenotype: Enhanced mutations of immunoglobulin and proto-oncogenes. Proc Natl Acad Sci USA 101:4262–4267.

Machida K, Cheng KT, Pavio N, Sung VM, Lai MM. 2005. Hepatitis C virus E2-CD81 interaction induces hypermutation of the immunoglobulin gene in B cells. J Virol 79:8079–8089.

Matsuura Y, Tani H, Suzuki K, Kimura-Someya T, Suzuki R, Aizaki H, Ishii K, Moriishi K, Robison CS, Whitt MA, Miyamura T. 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology 286:263–275.

Mazzaro C, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, Efremov DG, Burrone O, Santini G, Pozzato G. 1996. Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. Cancer 77:2604–2613.

Moldvay J, Deny P, Pol S, Brechot C, Lamas E. 1994. Detection of hepatitis C virus RNA in peripheral blood mononuclear cells of infected patients by in situ hybridization. Blood 83:269–273.

Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. 2004. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol 78:5867–5874.

Pozzato G, Burrone O, Baba K, Matsumoto M, Hijiiata M, Ota Y, Mazzoran L, Baracetti S, Zorat F, Mishiro S, Efremov DG. 1999. Ethnic difference in the prevalence of monoclonal B-cell proliferation in patients affected by hepatitis C virus chronic liver disease. J Hepatol 30:990–994.

Radkowski M, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, Wilkinson J, Adair D, Rakela J, Laskus T. 2005. Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology 41:106–114.

Ramos-Casals M, Brito-Zeron P, Yague J, Akasbi M, Bautista R, Ruano M, Claver G, Gil V, Font J. 2005. Hypocomplementaemia as an

immunological marker of morbidity and mortality in patients with primary Sjogren's syndrome. Rheumatology (Oxford) 44:89–94.

Sansonno D, Lauletta G, Dammacco F. 2004. Detection and quantitation of HCV core protein in single hepatocytes by means of laser capture microdissection and enzyme-linked immunosorbent assay. J Viral Hepat 11:27–32.

Schmidt WN, Stapleton JT, LaBrecque DR, Mitros FA, Kirby PA, Phillips MJ, Brashear DL. 2000. Hepatitis C virus (HCV) infection and cryoglobulinemia: Analysis of whole blood and plasma HCV-RNA concentrations and correlation with liver histology. Hepatology 31:737–744.

Sung VM, Shimodaira S, Doughty AL, Picchio GR, Can H, Yen TS, Lindsay KL, Levine AM, Lai MM. 2003. Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: The apoptotic effects of virus infection. J Virol 77:2134–2146.

Tong MJ, el-Farra NS, Reikes AR, Co RL. 1995. Clinical outcomes after transfusion-associated hepatitis C.N. Engl J Med 332:1463–1466.

Ulcickas Yood M, Quesenberry CP, Jr., Guo D, Caldwell C, Wells K, Shan J, Sanders L, Skovron ML, Iloeje U, Manos MM. 2007. Incidence of non-Hodgkin's lymphoma among individuals with chronic hepatitis B virus infection. Hepatology 46:107–112.

Weiner SM, Berg T, Berthold H, Weber S, Peters T, Blum HE, Hopf U, Peter HH. 1998. A clinical and virological study of hepatitis C virus-related cryoglobulinemia in Germany. J Hepatol 29:375–384.

Yanagi M, St Claire M, Shapiro M, Emerson SU, Purcell RH, Bukh J. 1998. Transcripts of a chimeric cDNA clone of hepatitis C virus genotype 1b are infectious in vivo. Virology 244:161–172.

Zignego AL, Brechot C. 1999. Extrahepatic manifestations of HCV infection: Facts and controversies. J Hepatol 31:369–376.

Zignego AL, Giannelli F, Marrocchi ME, Mazzocca A, Ferri C, Giannini C, Monti M, Caini P, Villa GL, Laffi G, Gentilini P. 2000. T(14;18) translocation in chronic hepatitis C virus infection. Hepatology 31:474–479.

Zignego AL, Ferri C, Pileri SA, Caini P, Bianchi FB. 2007. Extrahepatic manifestations of Hepatitis C Virus infection: A general overview and guidelines for a clinical approach. Dig Liver Dis 39:2–17.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Strong CD8$^+$ T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma

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Abstract
Aim We investigated whether tumor-specific CD8$^+$ T-cell responses affect tumor-free survival as well as the relationship between CD8$^+$ T-cell responses against tumor-associated antigens (TAAs) and the clinical course after tumor treatment in patients with hepatocellular carcinoma (HCC).
Methods Twenty patients with HCC that were treated by radiofrequency ablation or trans-catheter chemo-embolization (TACE) and in whom HCC was undetectable by ultrasonography, CT, and/or MRI 1 month after treatment were enrolled in the study. Before and after treatment for HCC, analyses of TAA (glypican-3, NY-ESO-1, and MAGE-1)-specific CD8$^+$ T-cell responses were evaluated with an interferon-γ enzyme-linked immunospot (ELISpot) assay using peripheral CD8$^+$ T-cells, monocytes, and 104 types of 20-mer synthetic peptide overlapping by 10 residues and spanning the entirety of the 3 TAAs.
Results Sixteen out of 20 patients (80%) showed a positive response ($\geq$10 TAA-specific cells/10^5 CD8$^+$ T-cells) before or after treatment. When we performed univariate analysis of prognostic factors for the tumor-free period in the 20 patients, platelet count, prothrombin time, and the number of TAA-specific CD8$^+$ T-cells after treatment were significant factors ($P = 0.027$, 0.030, and 0.004, respectively). In multivariate analysis, the magnitude of the TAA-specific CD8$^+$ T-cell response ($\geq$40 TAA-specific cells/10^5 CD8$^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free interval (hazard ratio 0.342, $P = 0.022$).
Conclusions Our results suggest that strong TAA-specific CD8$^+$ T-cell responses suppress the recurrence of HCC. Immunotherapy to induce TAA-specific cytotoxic T lymphocytes by means such as the use of peptide vaccines should be considered for clinical application in patients with HCC after local therapy.

Keywords Hepatocellular carcinoma · CD8$^+$ T-cell response · Cytotoxic T lymphocyte · ELISpot assay · Immunotherapy

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Introduction

There are about 500,000 new patients with hepatocellular carcinoma (HCC) per year worldwide. Although vaccination against hepatitis B virus (HBV) and interferon (IFN)-based therapy against hepatitis C virus (HCV) will presumably reduce the number of HCC patients in the future, the incidence of HCC is still increasing in Asia and Africa because of the previous prevalence of infection with the virus. Progress in treatments for HCC has improved the prognosis of patients with HCC. However, HCC is usually associated with cirrhosis and often recurs even after complete treatment of the tumors in the remaining part of the cirrhotic liver. Thus, there is a strong need for the development of a new intervention therapy that suppresses the occurrence or recurrence of HCC effectively and that has fewer side effects. Immunotherapy may be such a treatment and may be applicable to the clinical treatment of HCC. In fact, some clinical trials have been performed [1–3].

Cytotoxic T lymphocytes (CTLs) are thought to be potent effector cells against cancers. CTLs recognize specific antigens, and the induction of CTLs specific for tumor-associated antigen (TAA) is an attractive procedure

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for tumor therapy. The MAGE-1 gene was first identified as encoding a tumor-specific antigen on MZ-2-MEL cells, a melanoma cell line, in 1991 [4]. MAGE-1 gene and protein can be detected in many cancer tissues, and three articles reported the expression of MAGE-1 in HCC as 30, 68, or 78%, respectively, in a Japanese population [5–7]. In gastrointestinal tumors, immunotherapy using both dendritic cells and MAGE peptides has been performed for patients with primary malignant melanoma of the esophagus, and this therapy was able to induce peptide-specific immune responses [8].

NY-ESO-1 antigen, a member of the cancer-testis antigen family, was initially identified by a serological analysis of recombinant cDNA expression cloning in an esophageal cancer patient [9]. NY-ESO-1 mRNA was detected in 24–37% of HCCs by reverse transcription-polymerase chain reaction [10, 11].

Glypican-3 (GPC3) consists of 580 amino acids and is a heparan sulfate proteoglycan with a potential role in the control of cell division. GPC3 mRNA was detected in 74.8% of HCC tissues, but only in 3.2% of normal liver tissues [12], and GPC3 protein was detected in 72% of HCCs, but not in normal tissue using GPC-specific antibody [13]. The GPC3 protein can also be detected in sera of 40–53% of patients with HCC [14, 15].

These three antigens are thought to be attractive targets for cancer immunotherapy because they are expressed only in tumor tissues and testis, but not in normal tissues other than testis. On the basis of previous reports, it is assumed that most HCCs would express at least one of the three TAAs. Therefore, monitoring immune responses against these TAAs might help in the development of HCC immunotherapy, such as TAA-based vaccination. In this study, we investigated how the magnitude of $CD8^+$ T-cell responses against these TAAs determined by an IFN-γ enzyme-linked immunospot (ELISpot) assay is related to other clinical data and the tumor-free interval in patients with HCC, in order to explore the clinical application of such a TAA-based immunotherapy.

Methods

Patients

Twenty patients who were diagnosed with HCC at Showa University Hospital between 2006 and 2008 were enrolled in the study. They met the following study criteria: (1) pathologically confirmed as having HCC or a lesion with characteristic imaging features of HCC based on ultrasonography, CT, and/or MRI; (2) liver function classed as Child-Pugh A or B; (3) no extrahepatic metastasis or vascular invasion; (4) no previous or simultaneous cancers other than HCC; and (5) an indication for treatment such as radiofrequency ablation (RFA) or trans-catheter chemo-embolization (TACE). RFA was performed by well-trained hepatologists using usual methods according to previous reports [16]. A 16-gauge cooled-tip ablation electrode (Covidien, Boulder, CO) was used in the procedure. TACE was performed by well-experienced hepatologists and radiologists. A microcatheter was inserted from the femoral artery to the artery feeding the HCC superselectively after conventional hepatic angiography, and then a segmental or subsegmental TACE procedure was performed using gelatin, lipiodol, and either epirubicin hydrochloride or cisplatin. All patients were followed every 1–3 months by ultrasonography, CT, and/or MRI to examine the appearance of new lesions in the liver or other organs. The recurrence-free interval was defined as the period from the month of HCC treatment to the month when a recurrent and/or metastatic HCC was first detected after treatment. Clinical data (platelet count, prothrombin time, serum AST, ALT, albumin, total bilirubin level, and AFP level) were collected 1–7 days before HCC treatment. Chronic hepatitis C was diagnosed on the basis of detectable HCV RNA in serum using the Amplicor assay (Roche Diagnostics, Tokyo, Japan). Informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethical Committee of Showa University.

Synthetic peptides of TAA

Twenty-mer peptides overlapping by 10 residues and spanning the entire MAGE-1, NY-ESO-1, and GPC3 proteins were synthesized based on the amino acid sequences reported previously as PepSets™ and purchased from Mimotopes (Clayton South, Victoria, Australia). These peptides were >80% pure. A total of 30 MAGE-1, 17 NY-ESO-1, and 57 GPC3 peptides were synthesized, as shown in Table 1. A total of 10–11 TTA peptides were pooled in a mixture (total 10 mixtures) at a concentration of 10 μg/ml each.

Preparation of $CD8^+$ T cells and monocytes from patients with HCC

PBMCs were isolated from heparinized peripheral blood by gradient centrifugation using Ficoll-Paque (Pharmacia-LKB Biotechnology, Uppsala, Sweden). As reported previously, peripheral $CD8^+$ T-cells and monocytes were separated from PBMCs using CD8 microbeads (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany) and a Monocyte Isolation Kit II (Miltenyi Biotec), respectively [17]. These cells were isolated using an autoMACS™ Pro Separator (Miltenyi Biotec). The purity of the cells was >95% on flow cytometry (data not shown).

Table 1 Synthetic peptides and peptide mixtures used in this study

Tumor-associated antigen	Peptide	Amino acid sequence
Glypican-3	GL1	1–20
	GL2	11–30
	GL3	21–40
	⋮	⋮
	GL57	561–580
MAGE-1	MG-1	1–20
	⋮	⋮
	MG-30	290–309
NY-ESO-1	NY-1	1–20
	⋮	⋮
	NY-17	161–180

Mix 1	Mix 2	Mix 3	Mix 4	Mix 5	Mix 6	Mix 7	Mix 8	Mix 9	Mix 10
GL1	GL2	GL3	GL4	GL5	GL6	GL7	GL8	GL9	GL10
GL11	GL12	GL13	GL14	GL15	GL16	GL17	GL18	GL19	GL20
GL21	GL22	GL23	GL24	GL25	GL26	GL27	GL28	GL29	GL30
GL31	GL32	GL33	GL34	GL35	GL36	GL37	GL38	GL39	GL40
GL41	GL42	GL43	GL44	GL45	GL46	GL47	GL48	GL49	GL50
GL51	GL52	GL53	GL54	GL55	GL56	GL57	MG-1	MG-2	MG-3
MG-4	MG-5	MG-6	MG-7	MG-8	MG-9	MG-10	MG-11	MG-12	MG-13
MG-14	MG-15	MG-16	MG-17	MG-18	MG-19	MG-20	MG-21	MG-22	MG-23
MG-24	MG-25	MG-26	MG-27	MG-28	MG-29	MG-30	NY-1	NY-2	NY-3
NY-4	NY-5	NY-6	NY-7	NY-8	NY-9	NY-10	NY-11	NY-12	NY-13
NY-14	NY-15	NY-16	NY-17	–	–	–	–	–	–

IFN-γ ELISpot assay

The ELISpot assay was performed using an IFN-γ ELISpot assay kit (Mabtech AB, Stockholm, Sweden) as previously described [17]. Briefly, a 96-well microtiter plate with a nitrocellulose membrane bottom (Millititer; Millipore, Bedford, MA) was coated with 100 μl anti-IFN-γ monoclonal antibody at a concentration of 15 μg/ml in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by washing 6 times in Hanks' balanced saline solution. After blocking with AIM-V medium (Invitrogen Japan, Tokyo, Japan) containing 10% fetal bovine serum, 1×10^5 $CD8^+$ T-cells, 1×10^4 autologous monocytes, and a TAA peptide mixture at 10 μg/ml of each peptide were placed and incubated in duplicate in 100 μl AIM-V medium at 37°C in a humid atmosphere with 5% CO_2. After incubation for 18 h, the cells were removed by washing the plate 8 times with PBS. Next, 100 μl of biotin-conjugated monoclonal antibody was added to each well, and the plates were incubated further for 2 h at room temperature. Wells were washed 5 times with PBS and incubated with 100 μl streptavidin–alkaline phosphatase for 2 h. Unbound antibodies were removed by washing 6 times with PBS. Then, 100 μl of alkaline phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) was added to each well and incubated until dark spots emerged. Color development was stopped by washing 3 times with water, and the plates were allowed to dry. Using an ELISpot reader (KS ELISPOT compact; Carl Zeiss, Oberkochen, Germany), the number of spot-forming cells (SFCs) per well was counted. Numbers of TAA-specific SFCs for each peptide mixture were calculated by subtracting the mean number of SFCs of 2 control wells (without stimulus) from the mean number of SFCs of 2 wells stimulated by TAA antigens. An SFC number was calculated for each patient as the sum of SFCs in each peptide mixture. ELISpot assays were performed before and 3–7 days after treatment. When TAA-specific $CD8^+$ T-cell responses were analyzed in 10 normal subjects, we were unable to detect any responses against TAA peptides in the ELISpot assay (data not shown).

Statistical analyses

The relationship between the number of TAA-specific $CD8^+$ T-cells and the recurrence-free period was analyzed

using a parametric survival model. The log-rank test was used to compare recurrence-free data for 2 groups. The effects of multiple explanatory variables on recurrence-free interval were analyzed using a Cox proportional hazards model. Statistical analyses were performed using the statistical software JMP version 5 (SAS Institute Inc., Cary, NC). Differences were considered as significant when the P value was less than 0.05.

Results

TAA-specific $CD8^+$ T-cells were detected by ELISpot assay before and after HCC treatment in most HCC patients

The characteristics of the 20 patients enrolled in this study are shown in Table 2. The 20 patients had no HCC detected by ultrasonography, enhanced CT, and/or MRI 1 month after treatment for HCC. In those patients with HCCs who had up to 3 HCCs and in whom the diameter of each lesion was 3 cm or less, the treatment was usually RFA; the remaining patients were treated by TACE. However, in a few patients (patients 2 and 5) in whom the diameter of each lesion was less than 3 cm, the physician in charge of the patient selected TACE because they could not deny the existence of more lesions that were undetectable by conventional enhanced CT. The clinical courses of the patients were followed for 3–29 months after therapy for HCC. The ELISpot assay was performed to detect $CD8^+$ T-cell responses to TAAs before and 3–7 days after treatment. The data are shown in Table 3 as SFCs (total count of TAA-specific $CD8^+$ T-cells/1 $\times$ 10^5 $CD8^+$ T-cells). Sixteen out of 20 patients (80%) showed a positive response (10 or more SFCs) for TAA peptides either before and/or after treatment. The numbers of SFCs (mean $\pm$ SD) before and after therapy were 33.8 $\pm$ 51.4 (0–161, median 16.5) and 32.9 $\pm$ 34.7 (0–130, median 23.0), respectively. Of the 20 patients, 5 (25%) and 7 (35%) showed a high TAA-specific immune response (40 or more SFCs) before and after treatment, respectively.

When we analyzed the TAA peptides recognized by $CD8^+$ T-cells, we occasionally observed that different peptide mixtures were identified as positive before and after HCC treatment (data not shown).

Change in TAA-specific $CD8^+$ T-cell response induced by HCC treatment does not correlate with recurrence-free period

The number of SFCs increased in 11 of 20 (55%) patients after treatment. In these patients, TAA-specific CTLs might have been induced by the treatment. There were no significant differences between the increase in TAA-specific $CD8^+$ T-cell response induced by the treatment and either therapeutic procedure, laboratory data, or background of the patients (data not shown). The increase in TAA-specific CTLs after treatment did not predict a better prognosis of HCC.

Table 2 Characteristics of HCC patients before HCC treatment

	$n = 20$	Median
Age (years)[a]	68.8 $\pm$ 9.4	73.0
Gender		
M	11	
F	9	
AST (IU/l)[a]	70 $\pm$ 49	52
ALT (IU/l)[a]	63 $\pm$ 43	54
PLT ($\times 10^4/\mu l$)[a]	9.8 $\pm$ 5.3	8.5
PT (%)[a]	81 $\pm$ 11	78
Alb (g/dl)[a]	3.5 $\pm$ 0.4	3.4
T-Bil (mg/dl)[a]	0.9 $\pm$ 0.4	0.9
AFP (ng/ml)[a]	86 $\pm$ 157	16
Virus		
HCV	17	
NBNC	3	
Child-Pugh class		
A	12	
B	8	
HCC size (mm)[a]	23 $\pm$ 8	23
No. HCCs		
1	9	
2	4	
3	7	
>3	0	
Treatment		
RFA	13	
TACE	5	
RFA + TACE	2	

NBNC Negative for neither HBV nor HCV infection, *RFA* radiofrequency ablation, *TACE* trans-catheter chemo-embolization

[a] Results are shown as mean $\pm$ SD

Platelet count, prothrombin time, and the magnitude of TAA-specific immune response after treatment correlate with the recurrence-free period by univariate analysis

When we analyzed the relationship between TAA-specific SFCs detected by the ELISpot assay or other clinical variates and the HCC-free interval using a parametric survival model, we found that platelet count, prothrombin time, and the TAA-specific $CD8^+$ T-cell response after treatment significantly correlated with the HCC-free interval

Table 3 Results of IFN-γ ELISpot assay in patients in whom HCCs were not detected after therapy

Patient no.	SFC before treatment (/10^5 $CD8^+$ T-cells)	SFC after treatment (/10^5 $CD8^+$ T-cells)	Recurrence-free interval (month)
1	0	0	5
2	15	31	10
3	12	15	5
4	159	130	26
5	58	4	12
6	5	99	29[a]
7	15	17	7
8	20	41	7
9	135	9	12
10	1	6	3
11	8	9	6
12	10	57	15
13	34	42	13[a]
14	6	4	12[a]
15	23	8	9
16	59	37	12
17	12	29	23
18	161	72	24
19	18	4	15
20	25	44	23[a]

SFC Spot-forming cells

[a] These patients had no recurrence detected by ultrasonography, enhanced CT, and/or MRI after treatment

($P = 0.005$, 0.007, and 0.001, respectively). When univariate analysis of prognostic factors for the HCC-free interval was performed, only platelet count ($P = 0.027$; Fig. 1a), prothrombin time ($P = 0.030$; Fig. 1b), and the number of SFCs after treatment ($P = 0.004$; Fig. 1c) were found to be significant. Child-Pugh class A tended to prolong the HCC-free interval, although this was not significant ($P = 0.066$). The other factors, including the number of SFCs before treatment ($P = 0.407$), ALT level ($P = 0.644$), albumin level ($P = 0.488$), total bilirubin level ($P = 0.340$), HCC size ($P = 0.756$), HCC number ($P = 0.486$), and the procedure used for HCC treatment (RFA or TACE, $P = 0.481$), did not affect HCC-free survival, as confirmed by the log-rank test.

Multivariate analysis shows that the magnitude of TAA-specific $CD8^+$ T-cell responses correlates with the HCC-free interval after treatment in patients who have no detectable HCC after therapy

In a further analysis of the 20 patients with HCC who were treated by RFA or TACE and in whom no HCC was detectable 1 month after treatment, we performed multivariate analysis using a Cox proportional hazards model. On multivariate analysis, only the magnitude of TAA-specific $CD8^+$ T-cell responses (≥40 TAA-specific cells/10^5 $CD8^+$ T-cells) was the only significant prognostic factor for a prolonged tumor-free period after treatment

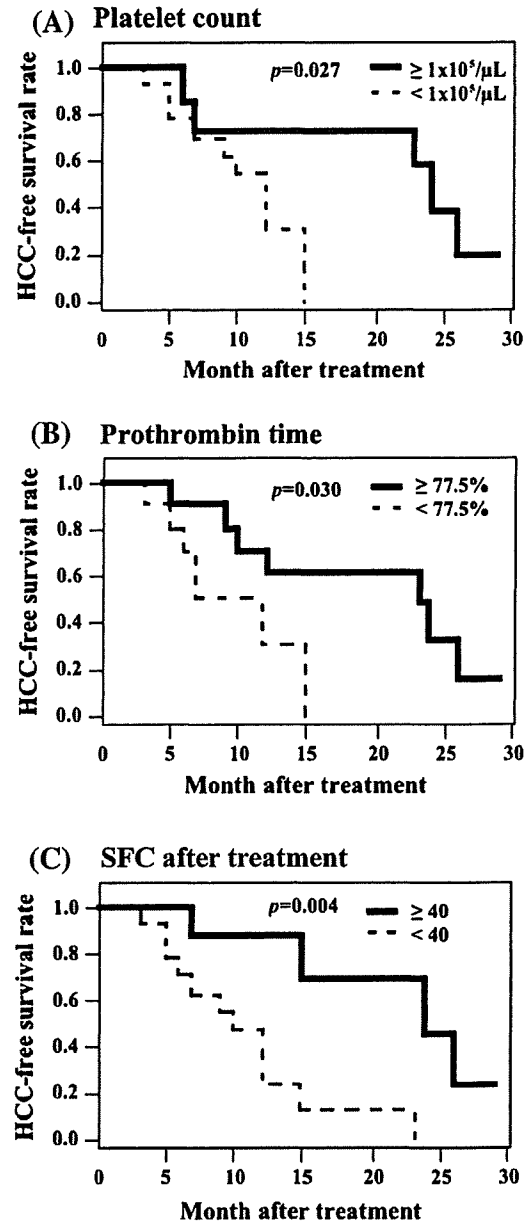

Fig. 1 Kaplan–Meier curves of HCC-free survival rate. In univariate analysis, platelet count, prothrombin time, and the tumor-associated antigen-specific $CD8^+$ T-cell response were found to be prognostic factors for the HCC-free period after treatment. Kaplan–Meier curves representing the relationship between month after treatment (HCC-free interval) and HCC-free survival rate were grouped by **a** platelet count, **b** prothrombin time, and **c** spot-forming cells (*SFCs*) specific for tumor-associated antigens after treatment

Table 4 Multivariate analyses of prognostic factors for tumor-free interval

Variable	Hazard ratio	95% Confidence limit	*P* value
Platelet count			
$\geq 1 \times 10^5/\mu L$	0.916	0.326–2.020	0.843
$< 1 \times 10^5/\mu L$	1.000		
Prothrombin time			
≥77.5%	0.455	0.094–1.390	0.177
<77.5%	1.000		
Child-Pugh class			
A	1.464	0.539–6.813	0.493
B	1.000		
Spot-forming cells after treatment			
≥40	0.342	0.079–0.866	0.022
<40	1.000		

(hazard ratio 0.342, $P = 0.022$), as shown in Table 4. Therefore, the results suggest that TAA-specific CTLs detected after treatment are able to suppress the occurrence or recurrence of HCC in patients with no detectable HCCs after treatment.

Discussion

To determine whether TAA-specific CTLs suppress the occurrence or recurrence of HCC, we investigated the relationship between the magnitude of TAA-specific $CD8^+$ T-cell responses and the HCC-free interval in patients who had no detectable viable HCC 1 month after treatment for HCC. We found that potent TAA-specific $CD8^+$ T-cell responses, as observed 1 month after treatment for HCC, led to a prolonged HCC-free interval.

An HLA-A24-restricted MAGE-1 peptide-specific CTL line was established in a patient with metastatic melanoma [18], and an NY-ESO-1 DNA vaccine induced both antigen-specific effector $CD4^+$ and/or $CD8^+$ T-cell responses in most patients who did not show detectable pre-vaccination immune responses [19]. In addition, HLA-A2- and HLA-A24-restricted GPC3-derived peptide vaccine induced specific CTLs in mice [20]. In this study, we selected GPC3, MAGE-1, and NY-ESO-1 to monitor antigen-specific $CD8^+$ T-cell responses against HCC because they had been reported to be expressed commonly and frequently in HCC tissues [7, 11–13], and thus the combination of these TAAs would cover most HCCs. Among the 20 patients enrolled in the present study, 16 (80%) showed positive $CD8^+$ T-cell responses (10 or more SFCs) against the TAAs before and/or after the treatment. Although we did not examine the expression of TAAs in the HCC tissues, it would be expected that at least one of these three TAAs will be expressed in HCCs in patients who have a positive $CD8^+$ T-cell response against TAAs.

In patient 10, HCC recurrence was detected 3 months after treatment. Insufficient treatment or the pre-existence of intrahepatic metastases might be considered in a patient in whom HCCs are undetectable 1 month after treatment, but are detected within a few months after treatment. We expected that TAA-specific CTLs induced by treatment would suppress the development of a small HCC, which is not easily detected by conventional methods of examination. Thus, we enrolled and analyzed all patients in whom no HCC was detectable by ultrasonography, CT, and/or MRI 1 month after treatment, even if a recurrent or metastatic HCC was detected within a few months after treatment.

It is of interest whether tumor destruction by local HCC treatment would induce immune responses against HCCs. Apoptotic tumor cells are capable of inducing tumor-specific immune responses [21]. Dendritic cells, representing antigen-presenting cells, around damaged tumor cells take up tumor antigen released from the tumor cells and then migrate into draining lymph nodes [22]. There, they mature and stimulate tumor-specific helper T-cells and CTLs. In turn, the effector cells migrate into the tumor tissue and attack the tumor cells [23]. Tumor-specific immune responses were induced by a combination of direct dendritic cell injections into the HCC and radiation therapy that might induce tumor destruction [3]. When we compared TAA-specific $CD8^+$ T-cell responses before HCC treatment and those after treatment, about half of the patients (55%) showed an increased frequency of TAA-specific $CD8^+$ T-cells, which might have been induced by the treatment. However, the increase in TAA-specific CTLs did not affect the recurrence-free interval. Rather, it was the magnitude of TAA-specific $CD8^+$ T-cell responses after the treatment itself that affected the recurrence-free interval. Even if the frequency of these CTLs seemed to be decreased after treatment, they might infiltrate the liver. Furthermore, new CTLs other than pre-existing CTLs might be induced by the treatment because many TAA peptides recognized by CTLs were different between before and after the treatment. Although some patients showed a potent TAA-specific $CD8^+$ T-cell response before treatment, SFC before treatment did not correlate with the recurrence-free interval. We believe that TAA-specific CTLs are not able to control a large tumor burden by itself. As HCCs enlarge, they may secret immune suppressive factors such as TGF-β [24] and/or IL-10 [25] and modify gene expression of TAAs [26]. We speculate that TAA-specific CTLs detected after the treatment, but not detected before the treatment may be able to control HCCs. Otherwise, TAA-specific CTLs detected before the

treatment may be able to destroy a small HCC that was not detected by conventional examinations.

The ELISpot assay is a convenient means of detecting antigen-specific $CD8^+$ T-cells in a variety of diseases. We have detected HCV-specific $CD8^+$ T-cell responses in patients with acute HCV infection using this method and identified 6 new epitopes within the HCV protein [17]. In fact, we identified a novel GPC3-specific CTL epitope using this method (unpublished observation). At present, we are trying to identify more CTL epitopes among these TAAs that will be used as cancer vaccines.

In this study, we used peptide mixtures to stimulate $CD8^+$ T-cells. This procedure may mask responses to individual peptides because a peptide that interacts only weakly with HLA molecules is unable to attach to the molecule if the mixture contains 1 peptide with a high affinity for the same molecule. However, such a weak peptide would not contribute to tumor immune responses because of its weak interaction with the HLA molecules. Thus, we ignored this issue in this study.

Recurrence and multicentric carcinogenesis are major factors in determining the prognosis of HCC, and several treatments have been tried for the prevention of recurrence. IFN therapy [27, 28], treatment with acyclic retinoid therapy [29, 30], and adoptive immunotherapy [31] have been reported as effective in suppressing HCC recurrence. Preoperative hepatic function influenced early HCC recurrence in patients in whom small HCCs were resected [32]. This is consistent with our result that prothrombin time, reflecting hepatic function, affected the recurrence-free interval in the univariate analysis. In our study, higher platelet counts also contributed to a longer recurrence-free interval in the univariate analysis. In the multivariate analysis, however, only the magnitude of TAA-specific $CD8^+$ T-cell responses remained as an independent factor contributing to a longer recurrence-free interval.

Although the size and number of HCCs were reported to affect the period of HCC-free survival (recurrence) in patients with HCC treated by hepatic resection [33], they are not significant factors affecting the recurrence-free interval. Further investigation, such as the accumulation of analyses of HCC patients, is needed to clarify this issue. Sixteen out of 20 patients without detectable HCC 1 month after treatment had recurrent or metastatic HCCs during the observation period in this study. Our results suggest that the maintenance of strong TAA-specific $CD8^+$ T-cell responses for a long period may lead to a longer recurrence-free state. A long-term observation of TAA-specific immune responses should also be performed in any future study.

The results of our study suggest that strong TAA-specific $CD8^+$ T-cell responses would suppress HCC recurrence in patients with HCC who are treated by RFA or TACE and in whom any HCC is undetectable by ultrasonography, CT, and/or MRI 1 month after treatment. Since recurrence and intrahepatic metastasis are major risk factors influencing the prognosis of patients with HCC, immunotherapy to induce TAA-specific $CD8^+$ T-cells, such as a peptide vaccine, should be considered for clinical application in patients with HCC after local therapy.

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References

1. Palmer DH, Midgley RS, Mirza N, Torr EE, Ahmed F, Steele JC, et al. A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009;49:124–32.
2. 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.
3. Chi KH, Liu SJ, Li CP, Kuo HP, Wang YS, Chao Y, et al. Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. J Immunother. 2005;28:129–35.
4. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science. 1991;254:1643–7.
5. Suzuki K, Tsujitani S, Konishi I, Yamaguchi Y, Hirooka Y, Kaibara N. Expression of MAGE genes and survival in patients with hepatocellular carcinoma. Int J Oncol. 1999;15:1227–32.
6. Tahara K, Mori M, Sadanaga N, Sakamoto Y, Kitano S, Makuuchi M. Expression of the MAGE gene family in human hepatocellular carcinoma. Cancer. 1999;85:1234–40.
7. Kariyama K, Higashi T, Kobayashi Y, Nouso K, Nakatsukasa H, Yamano T, et al. Expression of MAGE-1 and -3 genes and gene products in human hepatocellular carcinoma. Br J Cancer. 1999;81:1080–7.
8. Ueda Y, Shimizu K, Itoh T, Fuji N, Naito K, Shiozaki A, et al. Induction of peptide-specific immune response in patients with primary malignant melanoma of the esophagus after immunotherapy using dendritic cells pulsed with MAGE peptides. Jpn J Clin Oncol. 2007;37:140–5.
9. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc Natl Acad Sci USA. 1997;94:1914–8.
10. Korangy F, Ormandy LA, Bleck JS, Klempnauer J, Wilkens L, Manns MP, et al. Spontaneous tumor-specific humoral and cellular immune responses to NY-ESO-1 in hepatocellular carcinoma. Clin Cancer Res. 2004;10:4332–41.
11. Chen CH, Chen GJ, Lee HS, Huang GT, Yang PM, Tsai LJ, et al. Expressions of cancer-testis antigens in human hepatocellular carcinomas. Cancer Lett. 2001;164:189–95.
12. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular

carcinoma: biological significance and temporospatial distribution. Cancer Res. 1997;57:5179–84.
13. Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. Gastroenterology. 2003;125:89–97.
14. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun. 2003;306:16–25.
15. Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, et al. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res. 2004;64:2418–23.
16. Shiina S, Teratani T, Obi S, Sato S, Tateishi R, Fujishima T, et al. A randomized controlled trial of radiofrequency ablation with ethanol injection for small hepatocellular carcinoma. Gastroenterology. 2005;129:122–30.
17. Doi H, Hiroishi K, Shimazaki T, Eguchi J, Baba T, Ito T, et al. Magnitude of CD8 T-cell responses against hepatitis C virus and severity of hepatitis do not necessarily determine outcomes in acute hepatitis C virus infection. Hepatol Res. 2009;39:256–65.
18. Akiyama Y, Maruyama K, Tai S, Komiyama M, Iizuka A, Takikawa M, et al. Characterization of a MAGE-1-derived HLA-A24 epitope-specific CTL line from a Japanese metastatic melanoma patient. Anticancer Res. 2009;29:647–55.
19. Gnjatic S, Altorki NK, Tang DN, Tu SM, Kundra V, Ritter G, et al. NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells. Clin Cancer Res. 2009; 15:2130–9.
20. Motomura Y, Ikuta Y, Kuronuma T, Komori H, Ito M, Tsuchihara M, et al. HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. Int J Oncol. 2008;32:985–90.
21. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86–9.
22. Onaitis M, Kalady MF, Pruitt S, Tyler DS. Dendritic cell gene therapy. Surg Oncol Clin N Am. 2002;11:645–60.
23. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245–52.
24. Gastl G, Plante M, Finstad CL, Wong GY, Federici MG, Bander NH, et al. High IL-6 levels in ascitic fluid correlate with reactive thrombocytosis in patients with epithelial ovarian cancer. Br J Haematol. 1993;83:433–41.
25. Hersh EM, Stopeck AT. Advances in the biological therapy and gene therapy of malignant disease. Clin Cancer Res. 1997; 3:2623–9.
26. Cormier JN, Panelli MC, Hackett JA, Bettinotti MP, Mixon A, Wunderlich J, et al. Natural variation of the expression of HLA and endogenous antigen modulates CTL recognition in an in vitro melanoma model. Int J Cancer. 1999;80:781–90.
27. Ikeda K, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. Hepatology. 2000;32:228–32.
28. Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, et al. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. Ann Intern Med. 2001;134:963–7.
29. Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. N Engl J Med. 1996; 334:1561–7.
30. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. N Engl J Med. 1999;340:1046–7.
31. Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, et al. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. Lancet. 2000;356:802–7.
32. Kaibori M, Ishizaki M, Saito T, Matsui K, Kwon AH, Kamiyama Y. Risk factors and outcome of early recurrence after resection of small hepatocellular carcinomas. Am J Surg. 2009;198:39–45.
33. Maeda T, Shimada M, Harimoto N, Tsujita E, Aishima S, Tanaka S, et al. Prognosis of early hepatocellular carcinoma after hepatic resection. Hepatogastroenterology. 2008;55:1428–32.