Cancer Immunol Immunother (2008) 57:1861–1869
DOI 10.1007/s00262-008-0514-5

ORIGINAL ARTICLE

Dendritic cell-based vaccines suppress metastatic liver tumor via activation of local innate and acquired immunity

Shinjiro Yamaguchi · Tomohide Tatsumi · Tetsuo Takehara · Akira Sasakawa · Hayato Hikita · Keisuke Kohga · Akio Uemura · Ryotaro Sakamori · Kazuyoshi Ohkawa · Norio Hayashi

Received: 17 January 2008 / Accepted: 27 March 2008 / Published online: 26 April 2008

Abstract
Background Dendritic cell (DC)-based vaccines have been applied clinically in the setting of cancer, but tumor-associated antigens (TAAs) have not yet been enough identified in various cancers. In this study, we investigated whether preventive vaccination with unpulsed DCs or peptide-pulsed DCs could offer anti-tumor effects against MC38 or BL6 liver tumors.
Methods Mice were subcutaneously (s.c.) immunized with unpulsed DCs or the recently defined TAA EphA2 derived peptide-pulsed dendritic cells (Eph-DCs) to treat EphA2-positive MC38 and EphA2-negative BL6 liver tumors. Liver mononuclear cells (LMNCs) from treated mice were subjected to ^{51}Cr release assays against YAC-1 target cells. In some experiments, mice were injected with anti-CD8, anti-CD4 or anti-asialo GM1 antibody to deplete each lymphocyte subsets.
Results Immunization with unpulsed DCs displayed comparable efficacy against both MC38 and BL6 liver tumors when compared with Eph-DCs. Both DC-based vaccines significantly augmented the cytotoxicity of LMNCs against YAC-1 cells. In vivo antibody depletion studies revealed that NK cells, as well as, CD4+ and CD8+ T cells play critical roles in the anti-tumor efficacy associated with either DC-based modality.

Tumor-specific cytotoxic T lymphocyte (CTL) activity was generally higher if mice had received Eph-DCs versus unpulsed DCs. Importantly, the mice that had been protected from MC38 liver tumor by either unpulsed DCs or Eph-DCs became resistant to s.c. MC38 rechallenge, but not to BL6 rechallenge.
Conclusions These results demonstrate that unpulsed DC vaccines might serve as an effective therapy for treating *metastatic liver tumor*, for which TAA has not yet been identified.

Keywords Dendritic cells · Innate immunity · Liver tumor · Cancer immunotherapy

Abbreviations

DC	Dendritic cell
Eph-DCs	EphA2 derived peptide-pulsed dendritic cells
CTLs	Cytotoxic T cell lymphocytes
s.c.	Subcutaneously
SCID	Severe combined immuno-deficiency
BM	Bone marrow
GM-CSF	Granulocyte/macrophage-colony stimulating factor
PBS	Phosphate-buffered saline
LMNC	Liver mononuclear cell
TAA	Tumor-associated antigen

Shinjiro Yamaguchi and Tomohide Tatsumi contributed equally to this work.

S. Yamaguchi · T. Tatsumi · T. Takehara · A. Sasakawa · H. Hikita · K. Kohga · A. Uemura · R. Sakamori · K. Ohkawa · N. Hayashi (✉)
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
e-mail: hayashin@gh.med.osaka-u.ac.jp

S. Yamaguchi
e-mail: syamaguc@gh.med.osaka-u.ac.jp

T. Tatsumi
Medical Center for Translational Research, Osaka University Hospital, Osaka 565-0871, Japan

Introduction

Dendritic cell (DC)-based vaccines are attractive cancer modalities since DCs are competent to coordinately induce both tumor antigen-specific cytotoxic T lymphocytes (CTLs) and helper T cells [1–3]. In this regard, DCs pulsed with TAA derived peptides have proven clinically effective in eliciting protective and therapeutic anti-tumor immunity in the setting of a broad range of cancer types [3]. Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via IL-12 secretion and direct cellular interaction. The liver is an enriched source of innate immune cells such as NK cells and NKT cells compared with other organs, supporting the specialized role of this organ in the immune system [13–15]. Indeed, it has been shown that liver-associated innate immune cells play a critical role in the first-line defense against metastatic liver tumors [16, 17]. However, despite numerous reports supporting the efficacy of DC-based vaccines in murine s.c. tumor models [18–20], the efficacy of this approach in liver tumor models remains under developed. Given the possibility that DC-based vaccines may efficiently activate NK cells, NKT cells and specific T cells in the liver, they could offer a preferred immunotherapy for liver cancer.

The liver is the most common site of distal metastasis for tumors developing in distal organs, and physiologic status of this organ correlates with survival in patients with advanced disease, even if primary tumor site are resected curatively [21, 22]. Recently, adjuvant chemotherapies have been reported to yield significant improvement in disease (including liver metastasis) free interval and overall patient survival, however, dose-limiting toxicities were often observed and liver metastasis could not be completely prevented [23–25]. In contrast, several peptide-pulsed DC vaccines have been shown to be clinically capable of stimulating tumor-specific T cells in patients with tumor liver metastasis [26–28], suggesting that such treatments may represent a new strategy option in the setting of metastatic liver cancer. In this context, several tumor-associated antigen (TAA) peptides have been identified in various types of cancer that often metastasize to liver [29–31], however, this approach remains encumbered by the necessity to restrict patient accrual to those individuals harboring specific HLA types.

We previously demonstrated that the recently defined TAA EphA2 derived peptide-pulsed DC (Eph-DC) vaccine prevented the subcutaneous tumor growth in mice, but unpulsed DC vaccine did not [32]. In the present study, we examined the anti-tumor protection of Eph-DC vaccines in the liver tumor model, which is under the unique immunological environment. Unexpectedly, we observed that preventive vaccination with not only Eph-DCs but also unpulsed DCs provide anti-tumor protection as a result of the activation of both innate immune cells and specific T cells. This suggests that cultured autologous DC (alone or pulsed with TAA peptides) may represent an effective modality for patients with tumors localized to their livers.

Materials and methods

Mice

Female C57BL/6 mice and severe combined immuno-deficiency (SCID) mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at 6–8 weeks of age. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care and our study protocol complied with the institution's guideline.

Cell lines and culture

MC38, a mouse colon carcinoma cell derived from C57BL6/J mice, was generously provided by Dr. Kazumasa Hiroishi (Showa University School of Medicine, Tokyo, Japan). BL6, a melanoma cell line, and YAC-1, a sensitive cell line to NK cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cell lines were maintained in Complete Medium [RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin] at 37°C in 5% CO_2.

Generation of dendritic cells in vitro from bone marrow (BM) and DC-based peptide vaccines

The procedure used in this study was described previously [32]. Briefly, BM-DCs were separated by magnetic cell sorting using CD11c Micro Beads (Miltenyi Biotec) and typically represented >90% of the harvested population of the cells based on morphology and expression of the CD40, CD80, CD86 and MHC class II (data not shown). The H-2 K^b-binding $mEphA2_{682-689}$ epitope (VVSKYKPM) was kindly provided by Dr. Walter Storkus (University of Pittsburgh Cancer Institute). BM-DCs were incubated with the $mEph_{682-689}$ peptide at a concentration of 10 μg/ml/10^6 DC/ml CM for 2 h at 37°C. The cells were harvested and washed three times with phosphate-buffered saline (PBS) before use [20].

Animal experiments

C57BL/6 mice or SCID mice were immunized s.c. in the flank with 1×10^6 Eph-DCs or unpulsed DCs in a total

volume of 100 μl of PBS twice a week. On day 0, at the time of the second injection with Eph-DCs or unpulsed DCs, 2×10^6 MC38 cells (EphA2-positive) or 5×10^5 BL6 (EphA2-negative) tumor cells were inoculated intrahepatically. Mice were sacrificed 14 days after tumor inoculation and liver weight was measured. Data are reported as the average liver weight ± SD.

Cytolytic assays

Liver mononuclear cells (LMNCs) were isolated from the liver 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against NK-sensitive YAC-1 target cells. In some experiments, whole splenocytes were harvested 14 days after tumor inoculation, with T cells stimulated in vitro using MC38 cells pre-treated with Mitomycin C (Kyowa Hakko, Tokyo, Japan) in the presence of 30 IU/ml murine IL-2 (Strathmann Biotech, Hannover, Germany) for 5 days. Lymphocytes (bulk, CD4-depleted, or CD8-depleted) were then harvested and analyzed for their ability to kill MC38 tumor cells in 4-h ^{51}Cr-release assays.

Flow cytometric analysis

Liver mononuclear cells were isolated from the liver prior to the first vaccination and on days 1, 3, 7 after tumor inoculation as previously described [16]. The phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. NK and NKT cells in the liver were identified separately by using PE anti-mouse NK1.1 monoclonal antibody and FITC anti-mouse TCR (BD Pharmingen, San Diego, CA, USA). Furthermore, NK activation was examined by using FITC anti-mouse CD69, the early activation marker (BD Pharmingen). Analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as NK1.1+/TCR-lymphocytes and NKT cells as NK1.1+/TCR+ lymphocytes. Activated NK1.1+ cells were identified as NK1.1+/CD69+ lymphocytes.

In vivo depletion experiments

The procedure used in this study was described previously [32]. The efficiency of specific subset depletions (CD4+, CD8+ T cell or NK cell) was comfirmed by flow cytometric analysis. In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Tumor rechallenge

C57BL/6 mice were immunized s.c. with 1×10^6 Eph-DCs or unpulsed DCs twice a week and then challenged intrahepatically with 2×10^6 MC38 cells, at the time of the second Eph-DC or unpulsed DC immunization. On day 14 after tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. in the flank. As a control, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into naïve C57BL/6 mice. Tumor size was assessed on a weekly basis and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± SD.

Statistical analyses. Statistical differences between the groups was determined by applying a Student's t test with Welch correction or one-way ANOVA after each group had been tested with equal variance and Fisher's exact probability test. Statistical significance was defined as $P < 0.05$.

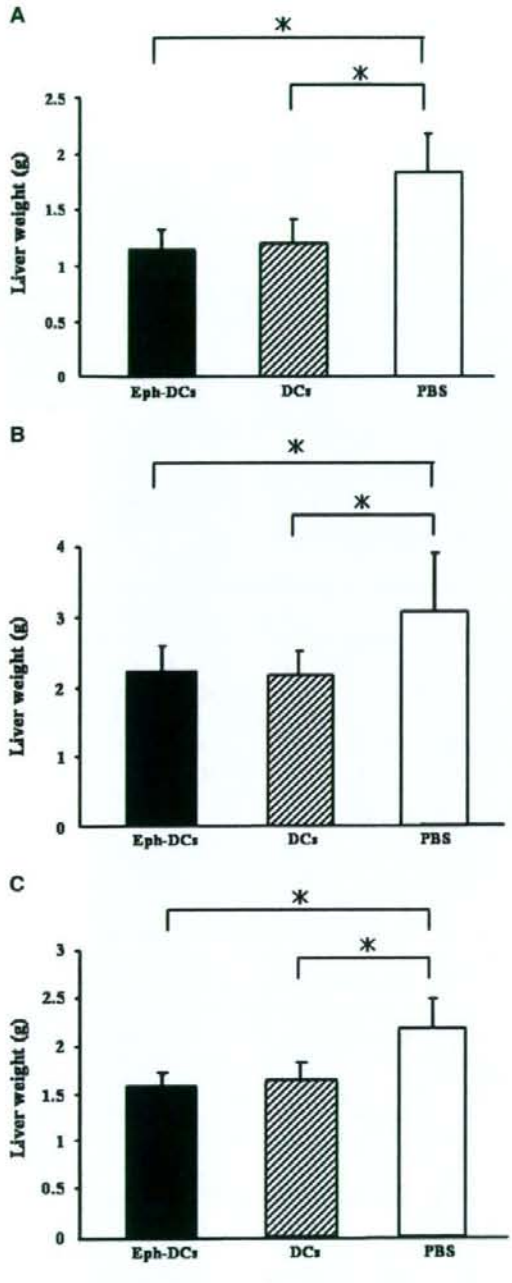
Results

Immunization with Eph-DCs or unpulsed DCs prevents progression of liver tumors in vivo

We examined whether immunization with Eph-DCs or unpulsed DCs would promote protective anti-tumor effects against the EphA2-positive MC38 or EphA2-negative BL6 liver tumors. MC38 liver tumor growth in mice immunized with either Eph-DCs or unpulsed DCs was significantly inhibited when compared to mice treated with PBS. Immunization with unpulsed DCs provided an equitable degree of anti-MC38 protection to that observed for immunization using Eph-DCs (Fig. 1a). BL6 tumor growth was also significantly inhibited by Eph-DCs or unpulsed DCs, to a comparable degree (Fig. 1b). These results suggest that immunization with DCs (whether pulsed with peptide or not) successfully inhibits the growth of two distinct H-2^b tumors established in the liver. Moreover, MC38 liver tumor growth in SCID mice (T cell, B cell and NKT cell deficient mice) immunized with DCs (either Eph-DCs or unpulsed DCs) was also significantly inhibited when compared to PBS treated mice, with no significant difference between the Eph-DC and the unpulsed DC groups (Fig. 1c). These results suggest that hepatic NK cells play an important role in regulating tumor growth in the liver after being activated by DC-based vaccination.

Liver NK cells are activated by DC vaccination

We examined whether LMNCs isolated from the liver 1 day after tumor inoculation displayed increased cytolytic activity against YAC-1 target cells in vitro. LMNCs harvested from mice treated with DCs (±peptide) were better killers of YAC1 cells than control LMNCs from PBS-treated or naïve mice (Fig. 2a). In contrast, splenocytes harvested from these same animals displayed only weak



◀ **Fig. 1** Anti-tumor effects with DC-based vaccination against liver and lung tumor. C57BL/6 mice were immunized on day-7 and 0 with 1×10^6 Eph-DCs, unpulsed DCs or PBS. On day 0, 2×10^6 MC38 cells (**a**) or 5×10^5 BL6 cells (**b**) were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$; $N = 10$/group. *Each data point* represents the mean liver weight ± SD. **c** SCID mice that lack T cells, B cells and NKT cells were immunized with 1×10^6 Eph-DCs, unpulsed DCs or PBS on day-7 and 0. On day 0, 2×10^6 MC38 cells were injected intrahepatically. Mice were sacrificed and liver weight was then determined 14 days after tumor inoculation, $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

anti-YAC1 killing capacity (Fig. 2b). These results suggest the preferential activation of liver versus splenic NK effector cells by DC-based vaccination. We next examined the activation status (expression of CD69) of NK1.1+ cells by flow cytometry after DC-based vaccination. The frequency of hepatic NK1.1+ cells in mice immunized with Eph-DCs or unpulsed DCs were equal to those noted for mice treated with PBS alone (data not shown). The CD69 expression level on NK1.1+ cells in mice treated with either form of DC-based vaccine was significantly stronger than that of mice treated with PBS on day 1 after tumor inoculation, with this level of expression decreasing gradually on days 3, 7 after tumor inoculation (Fig. 2c). These results suggested that hepatic NK1.1+ cells were efficiently activated by DC vaccination versus PBS treatment. NK cells isolated from mice treated with Eph-DCs or unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment, and there is no difference in anti-tumor killing by liver NK cells between Eph-DC group and unpulsed DC group (Fig. 2d).

Depletion of CD4+ T cells, CD8+ T cells or NK cells impairs the anti-tumor effects of immunization with DCs

To prove whether the therapeutic benefit associated with DC vaccination in the MC38 liver tumor model was dependent on CD4+, CD8+ T cells or NK cells in vivo, we performed selective cell subset depletion studies. The anti-tumor efficacy of DC-based immunization was significantly reduced in CD4+, CD8+ T cell or NK cell-depleted mice (Fig. 3). Notably, the liver weights of NK cell-depleted mice were significantly heavier than those of CD8+ T cell-depleted mice if the animals received unpulsed DC injections (Fig. 3a), while this was not observed for mice injected with Eph-DCs (Fig. 3b). These results suggest that not only NK cells, but also CD4+ T cells and CD8+ T cells are required for optimal anti-tumor effects associated with either DC vaccines, but that NK cells may play a greater role than CD8+ T cells in regulating tumor growth in mice receiving unpulsed DCs.

Induction of specific CTLs against MC38 cells after immunization with DCs

We next examined whether either Eph-DC or unpulsed DC immunization induced specific splenocyte (harvested 14 days after tumor inoculation) cytolytic activity against

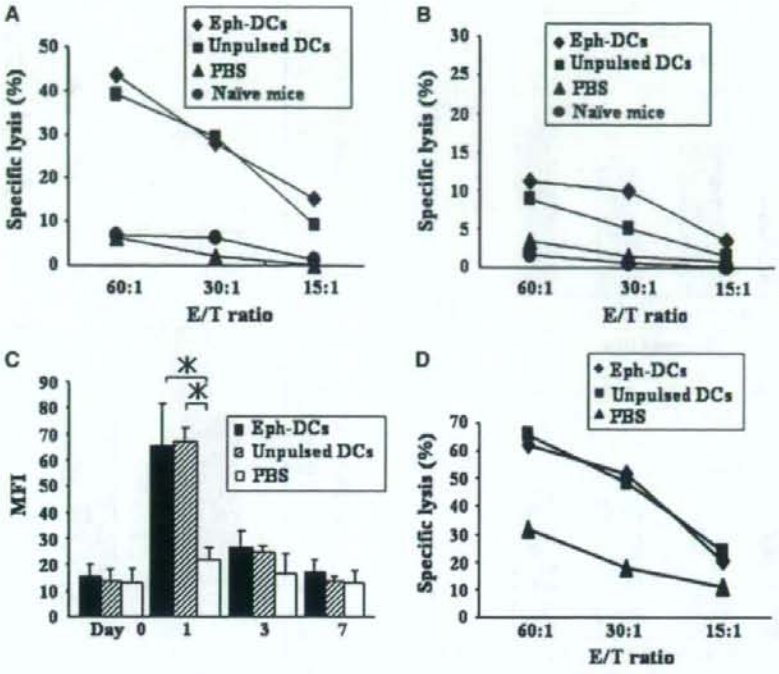


Fig. 2 Liver NK cells are activated by DC-based vaccination. LMNCs (**a**) or splenocytes (**b**) were isolated from the various treatment groups (Eph-DCs, unpulsed DCs or PBS) of mice or naïve mice 1 day after tumor inoculation, and subjected to 4-h ^{51}Cr release assays against YAC-1 targets at the indicated E:T ratios. Similar results were obtained in three experiments. **c** LMNCs were harvested before the first vaccination and on days 1, 3, 7 after tumor inoculation, and the phenotype of LMNCs from mice treated with Eph-DCs, unpulsed DCs or PBS was examined by flow cytometric analysis. Activated NK1.1 + cells were identified as NK1.1 + /CD69 + lymphocytes. *MFI* mean fluorescence intensity, $N = 3$/group. **d** LMNCs were isolated from the various treatment groups of mice 1 day after tumor inoculation, and liver NK cells were isolated from LMNCs by magnetic cell sorting using DX-5 MicroBeads (Miltenyi Biotec) and then subjected to 4-h ^{51}Cr release assays against MC38 target cells

MC38 or BL6 cells. Splenocytes isolated from mice treated with unpulsed DCs displayed stronger cytolytic activity against MC38 targets when compared with PBS treatment. Furthermore, splenocytes harvested from mice treated with Eph-DCs displayed stronger anti-MC38 cytolytic activity than unpulsed DC or PBS group (Fig. 4a), with this activity mediated by CD8+ T cells, but not CD4+ T cells (Fig. 4b). Cytolytic activity was not observed against EphA2-negative cells (i.e. BL6; Fig. 4c). These results suggest that either format of DC immunization induces MC38-specific CTLs in vivo, with somewhat greater levels of response observed in the case of peptide-specific vaccination.

Immunity to tumor rechallenge

We next determined whether DC vaccines that slow liver tumor progression protected mice against a consequent s.c. rechallenge with that same tumor. C57BL/6 mice were s.c. immunized with Eph-DCs or unpulsed DCs and MC38 liver tumors implanted. On day 14 post-intrahepaic tumor inoculation, 2×10^5 MC38 or 5×10^4 BL6 cells were injected s.c. into the flank of these mice and tumor growth monitored. We observed that s.c. MC38 (EphA2-positive) was inhibited by prior vaccination with Eph-DCs > unpulsed DCs versus naïve mice (Fig. 5a), and that the growth of BL6 (EphA2-negative) was not inhibited in any cohort analyzed versus control (Fig. 5b). These results suggested that immunization with either format of DCs elicited some degree of systemic anti-tumor effects against the EphA2-positive tumor, but that the specific immunization (Eph-DCs) was superior to unpulsed DCs in generating protective effects against EphA2-positive tumor outside the liver.

Discussion

DCs pulsed with TAA derived peptides (Peptide-DCs) have proven effective in eliciting protective and therapeutic anti-tumor immunity in patients against a diverse range of

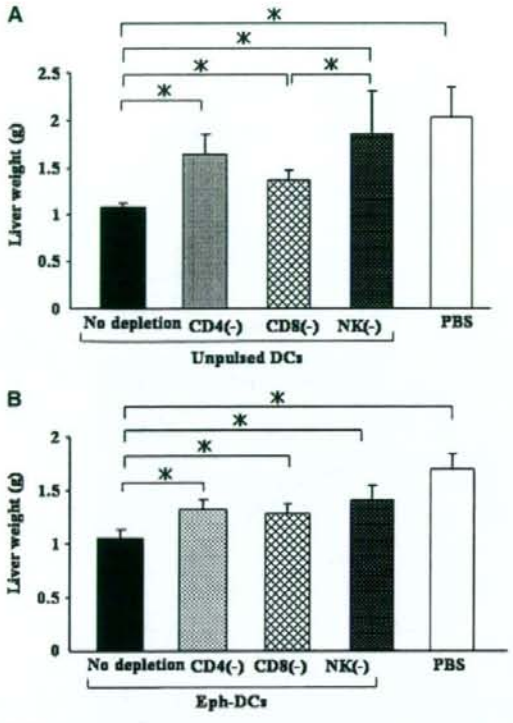


Fig. 3 DC-based vaccine efficacy is dependent upon T cells and NK cells. Ab-mediated in vivo depletion of CD4+, CD8+ T cells and NK cells were performed (as described in "Materials and methods"), with the depleted mice then receiving unpulsed DC (**a**) or Eph-DC vaccines (**b**) (on day-7 and 0) and intrahepatic 2×10^6 MC38 cell injection (day 0), $^*P < 0.05$, $N = 8$/group. *Each data point* represents the mean liver weight ± SD

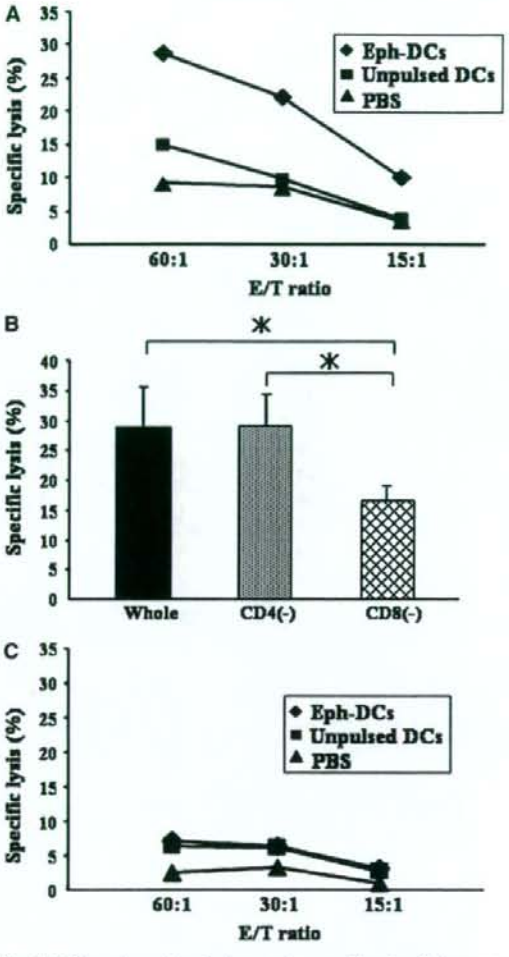


Fig. 4 DC-based vaccines induce anti-tumor T cells. Splenocytes were harvested from tumor-bearing mice 14 days after final treatment with Eph-DCs, unpulsed DCs or PBS. Splenocytes were stimulated in vitro with MMC-treated MC38 cells in the presence of low-dose recombinant human IL-2. After 5 days of culture, the cytolytic activity of the expanded T cells was evaluated using 4-h ^{51}Cr release assays against MC38 (**a**) or irrelevant BL6 (**c**) tumor target cells at the indicated E:T ratios. **b** Before performance of 4-h ^{51}Cr release cytolytic assays, CD4+ or CD8+ T cells were depleted from whole splenocytes of Eph-DC treated mice using specific MicroBeads. Similar results were obtained in three independent experiments

cancers [3]. However, at present, peptide-DC vaccines have not been comprehensively evaluated in clinical trials for treatment of metastatic liver cancers [25–27]. The liver uniquely contains an abundance of not only T cells, but also NK cells and NKT cells when compared with other organs [13–15]. Recently, DCs have been implicated as playing an important role in the activation of NK and NKT cells in both mice and humans [4–12, 33, 34], suggesting that DC-based therapies would be poised to activate an array of innate immune effector cells in the liver and might mediate clinical benefit within that organ. In this study, we demonstrated that administration of DCs prior to tumor implantation successfully promoted protective anti-tumor immunity against two distinct tumors in the liver (with little requirement for antigen-loading of DCs). Moreover, either Eph-DCs or unpulsed DCs have also proven effective in eliciting equally protective anti-tumor immunity against BL6 metastasis models in the lung that contains relatively high frequencies of innate immune effector cells (unpublished data).

In vitro cytotoxicity assays performed against the YAC-1 target cell line revealed that hepatic (and to a lesser extent splenic) NK cells were activated and mediated stronger killing function as a result of mice being treated with DCs (unpulsed or EphA2 peptide-pulsed) vaccination versus PBS controls. Consistent with this functional finding, hepatic NK 1.1+ cells (probably NK and NKT cells) in DC

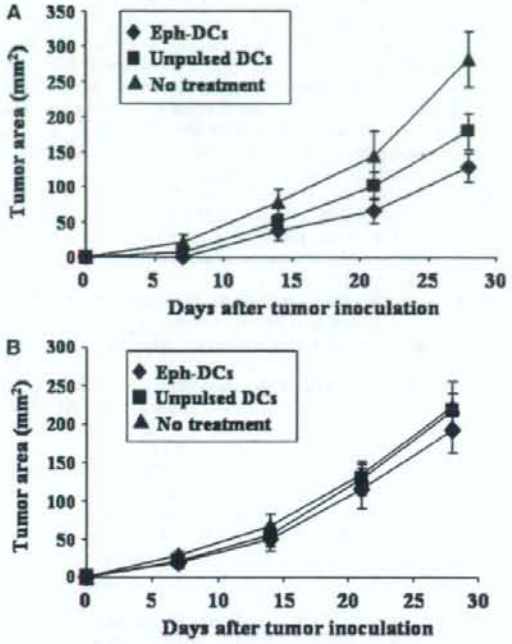


Fig. 5 DC vaccinated mice are partially protected against subcutaneous rechallenge with MC38 (EphA2-positive) but not BL6 (EphA2-negative) tumors. MC38-bearing mice that were successfully treated with DC-based vaccines were rechallenged s.c. in the flank with MC38 (**a**) or BL6 (**b**) tumor cells 14 days after the final DC injection. As controls, naïve mice were injected s.c. with MC38 or BL6 tumor cells. MC38 tumor growth in mice immunized with either format of DCs was significantly inhibited when compared to naive mice ($P < 0.05$ on day 28). In addition, tumor growth in the mice immunized with Eph- DCs was inhibited to a greater extent; i.e. $P < 0.05$ on day 28 versus unpulsed DCs, $P < 0.05$ on days 14, 21, 28 versus naive mice, $N = 8$/group. **b** Growth of the EphA2-negative BL6 tumor was not inhibited regardless of the treatment received, $N = 8$/group

treated mice also expressed elevated levels of the activation marker CD69 when compared to control treated animals.

Overall, these results suggest organ (i.e. Liver) focused anti-tumor "clinical" benefit derived from DC administration and that in conjunction with our results obtained in SCID models (lacking B cells, T cells, NKT cells) and in vitro cytotoxicity assays of liver NK cells performed against MC38 target cells, NK cells are major mediators of hepatic protection against tumor progression.

Recent studies have also suggested that DCs may effectively activate elements of innate immunity (NK cells [4–9] and NKT cells [10–12]) via direct cellular interaction and IL-12 secretion. In this study, the injected green fluorescence protein (GFP) mice-derived BM-DCs did not migrate to the liver following s.c. injection.in the flow cytometric analysis and IL-12 production in the serum harvested 1 day after either DC vaccination and tumor inoculation was not detected using IL-12 ELISA kits (unpublished data). Therefore, we would speculate that the injected DCs effectively enhance NK cell activity in the liver by some unknown humoral factors (cytokines except for IL-12, etc) or by secondary activated immune cells after DC immunization in vivo.

However, it is also clear that based on our in vivo lymphocyte depletion studies, CD4+ T cells and CD8+ T cells also contribute to suppression of tumor growth in the liver after DC vaccination. We would therefore speculate that hepatic NK cells may be a crucial early mediators of anti-tumor activity, with MC38 liver tumor-derived antigens then taken up by dedicated professional antigen-presenting cells in the liver that consequently cross-prime MC38 tumor-specific CD4+ or CD8+ T cells. In animals pre-vaccinated with Eph-DCs, the initial wave of tumor killing by NK cells may be boostered by EphA2-specific CD8+ T cells in the MC38 (EphA2-positive) model. This added degree of protection appeared to be dependent solely upon the presence of the loaded antigenic peptide on DCs, since we observed no other differences in Eph-DCs versus unpulsed DCs with regard to DC expression of a broad range of markers/parameters: CD40, CD80, CD86, MHC class II and IL12 production (S. Yamaguchi, unpublished data).

Data from our tumor rechallenge experiments indicate that unpulsed DC vaccines are not only effective in limiting MC38 liver tumor progression, but also offer protection against reintroduction of MC38 tumor at an extrahepatic site. However, Eph-DC immunizations were more effective in this endpoint, consistent with their superior capacity to promote tumor (i.e. EphA2)-specific CTLs. In contrast, in all cases when animals were rechallenged with the unrelated, EphA2-negative BL6 tumor, lesions grew progressively, suggesting that MC38-specific immunity was generated after liver tumor treatment. These results demonstrated that both unpulsed DC and peptide-pulsed DC vaccines generated systemic tumor-specific anti-tumor immunity against *metastatic liver tumor*, and that peptide-pulsed DC vaccination offered more optimal preventive treatment for extrahepatic tumor recurrence than unpulsed DC vaccines.

In spite of recent progress and early success reported for adjuvant chemotherapy trials in the prevention of liver metastasis, there remains a great need for developing novel and effective treatment modalities for this indication. In the current study, we have demonstrated that unpulsed DC vaccines, that are competent to activate both innate and acquired immunity within the liver, may represent a novel treatment option for metastatic liver tumors which expressed unknown TAAs. In cases where more disseminated disease is also present (or likely to occur) in non-liver

sites, the greatest degree of clinical efficacy may be expected of Peptide-DC vaccines, such as Eph-DCs.

Acknowledgments The authors wish to thank Dr. Walter J. Storkus (The University of Pittsburgh) for careful review and comments provided during the preparation of this manuscript. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labor and Welfare of Japan [N.H.].

References

1. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271–296
2. Hart DN (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90:3245–3287
3. Smith CM, Wilson NS, Waithman J, Villadangos JA, Carbone FR, Heath WR, Belz GT (2004) Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nat Immunol 5:1143–1148
4. O'Neill DW, Adams S, Bhardwaj N (2004) Manipulating dendritic cell biology for the active immunotherapy of cancer. Blood 104:2235–2246
5. Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5:405–411
6. Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G (2002) Reciprocal activating interaction between natural killer cells and dendritic cells. J Exp Med 195:327–333
7. Miller G, Lahrs S, Dematteo RP (2003) Overexpression of interleukin–12 enables dendritic cells to activate NK cells and confer systemic antitumor immunity. FASEB J 17:728–730
8. Piccioli D, Sbrana S, Melandri E, Valiante NM (2002) Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J Exp Med 195:335–341
9. Jinushi M, Takehara T, Kanto T, Tatsumi T, Groh V, Spies T, Miyagi T, Suzuki T, Sasaki Y, Hayashi N (2003) Critical role of MHC class I-related chain A and B expression on IFNα-stimulated dendritic cells in NK cell activation: Impairment in chronic hepatitis C virus infection. J Immunol 170:1249–1256
10. Kawano T, Cui J, Koezuka Y, Toura Y, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M (1997) CD1d-restricted and TCR-mediated activation of Vα14NKT cells by glycosylceramids. Science 278:1626–1629
11. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Tanaka Y, Taniguchi M (1998) Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Vα14NKT cells. Proc Natl Acad Sci USA 95:5690–5693
12. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, Sato M, Takeda K, Okumura K, Van Kaer L, Kawano T, Taniguchi M, Nishimura T (1999) The natural killer T (NKT) cell-ligand-Galactosylceramide demonstrates immunopoteniating effects by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. J Exp Med 189:1121–1128
13. Wiltrout RH (2000) Regulation and antimetastatic functions of liver-associated natural killer cells. Immunol Rev 174:63–76
14. George AP, Catherine AP (2005) Liver immunobiology. Toxicol Pathol 33:52–62
15. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in human liver. Immunol Rev 174:5–20
16. Miyagi T, Takehara T, Tatsumi T, Kanto T, Suzuki T, Jinushi M, Sugimoto Y, Sasaki Y, Hori M, Hayashi N (2003) CD1d-mediated stimulation of natural killer T cells selectively activates hepatic natural killer T cells to eliminate experimentally disseminated hepatoma cells in murine liver. Int J Cancer 106:81–89
17. Nakagawa R, Motoki K, Ueno H, Iijima R, Nakamura H, Kobayashi E, Shimosaka A, Koezuka Y (1998) Treatment of hepatic metastasis of the colon 26 adenocarcinoma with a Galactosylceramide, KRN7000. Cancer Res 58:1202–1207
18. Mayordome JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Kast WM, Deleo AB et al (1995) Bone marrow-derived dendritic cells pulsed with synthetic tumor peptide elicits protective and therapeutic antitumor immunity. Nat Med 12:1297–1302
19. Paglia P, Chiodoni C, Rodolfo M, Colombo MP (1996) Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med 183:317–322
20. Mayordomo JI, Loftus DJ, Sakamoto H, De Cesare CM, Appasamy PM, Lotze MT, Storkus WJ, Appella E, DeLeo AB (1996) Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J Exp Med 183:1357–1365
21. Olson RM, Perencevich NP, Malcolm AW, Chaffey JT, Wilson RE (1980) Patterns of recurrence following curative resection of adenocarcinoma of the colon and rectum. Cancer 45:2969–2974
22. Malcolm AW, Perencevich NP, Olson RM, Hanley JA, Chaffey JT, Wilson RE (1981) Analysis of recurrence patterns following curative resection for carcinoma of thecolon and rectum. Surg Gynecol Obstet 152:131–136
23. Ota M, Shimada H, Masui H, Tanaka K, Yamaguchi S, Ichikawa Y, Togo S, Ike H, Oki S (2004) Adjuvant hepatic arterial infusion chemotherapy after curative resection for Dukes colorectal cancer: a pilot study. Hepatogastroenterology 51:124–127
24. Arkenau HT, Rettig K, Porschen R (2005) Adjuvant chemotherapy in curative resected colon carcinoma: 5-fluorouracil/leucovorin versus high-dose 5- fluorouracil 24-h infusion versus high-dose 5-fluorouracil 24-h infusion. Int J Colorectal Dis 20:258–261
25. Hayashibe A, Kameyama M, Shinbo M, Makimoto S (2007) Clinical results on intra-arterial adjuvant chemotherapy for prevention of liver metastasis following curative resection of pancreatic cancer. Ann Surg Oncol 14:190–194
26. Liu KJ, Wang CC, Chen LT, Cheng AL, Lin DT, Wu YC, Yu WL, Hung YM, Yang HY, Juang SH, Whang-Peng J (2004) Generation of carcinoembryonic antigen (CEA)- specific T-cell responses in HLA-A*0201 and HLA-A*2402 late-stage colorectal cancer patients after vaccination with dendritic cells loaded with CEA peptides. Clin Cancer Res 10:2645–2651
27. Sadanaga N, Nagashima H, Mashino K, Tahara K, Yamaguchi H, Ohta M, Fujie T, Tanaka F, Inoue H, Takesako K, Akiyoshi T, Mori M (2001) Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. Clin Cancer Res 7:2277–2284
28. Timmerman JM, Levy R (1999) Dendritic cell vaccines for cancer immunotherapy. Annu Rev Med 50:507–529
29. Nevellino L, Castelli C, Parmiani G (2005) A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother 54:187–207
30. Simone M, Carlo RR, Mario L, Donato N (2004) Colorectal cancer vaccines: principles, results, and perspectives. Gastroenterology 27:1821–1837
31. Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H, Elisseeva OA, Oji Y, Kawakami M, Ikegame K, Hosen N, Yoshihara S, Wu Y, Fujiki F, Murukami M, Masuda T, Nishida S, Shirakata T, Nakatsuka S, Sasaki A, Udaka K, Dohy H, Aozasa K, Noguchi S, Kawase I, Sugiyama H (2004) Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine

and the resultant cancer regression. Pro Natl Acad Sci USA 138:13885–13890
32. Yamaguchi S, Tatsumi T, Takehara T, Sakamori R, Uemura A, Mizushima T, Ohkawa K, Hayashi N (2007) Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines. Cancer 110(7):1469–1477
33. Felazzo G, Munz C (2004) NK cell compartments and their activation by dendritic cells. J Immunol 172:1333–1339
34. Nieda M, Okai M, Tazbirkova A, Lin H, Yamamura A, Ide K, Abraham R, Juji T, Macfarlane DJ, Nicol AJ (2004) Therapeutic activation of Vα24 + Vβ11 + NKT cells in human subjects results in highly coordinates secondary activation of acquired and innate immunity. Blood 103:383–389

ARTICLE | Annals of Internal Medicine

Declining Incidence of Hepatocellular Carcinoma in Osaka, Japan, from 1990 to 2003

Hideo Tanaka, MD; Yasuharu Imai, MD; Naoki Hiramatsu, MD; Yuri Ito, PhD; Kazuho Imanaka, MD; Masahide Oshita, MD; Taizo Hijioka, MD; Kazuhiro Katayama, MD; Iwao Yabuuchi, MD; Harumasa Yoshihara, MD; Atsuo Inoue, MD; Michio Kato, MD; Tetsuo Takehara, MD; Shinji Tamura, MD; Akinori Kasahara, MD; Norio Hayashi, MD; and Hideaki Tsukuma, MD

Background: Japan has the highest incidence rate of primary liver cancer attributed to chronic hepatitis C virus (HCV) infection among developed countries. Molecular clock analysis of HCV sequences revealed that the spread of HCV took place earlier in Japan than in other countries. This might influence recent temporal trends in hepatocellular carcinoma (HCC) incidence.

Objective: To characterize the contribution of HCV-related hepatocellular carcinoma (HCC) to recent changes in HCC incidence in Osaka, Japan.

Design: Population-based survey.

Setting: Osaka Cancer Registry and 10 hospitals in Osaka.

Participants: 63 862 patients with HCC that was diagnosed between 1981 and 2003 in Osaka Prefecture, including 5253 HCV-seropositive patients with HCC that was diagnosed between 1990 and 2003 at 10 hospitals.

Measurements: Incidence of HCC and estimated incidence rate of HCV-related HCC, measured by multiplying the prevalence of anti-HCV by the corresponding HCC incidence rate.

Results: Between 1981 and 2003, peak incidence of HCC among men age 50 to 59 years, 60 to 69 years, and 70 to 79 years occurred in 1986, 1995, and 2000, respectively, with marked downward trends thereafter (average annual change, −7.9, −22.3, and −12.4 per 100 000 persons, respectively). Similar trends were observed in women. Estimated sex- and age-specific incidence of HCV-related HCC (per 100 000 persons) decreased from 255 to 92 cases at the maximum in men age 60 to 69 years and from 61 to 34 cases in women age 60 to 69 years, whereas estimated incidence of non-HCV–related HCC did not change between 1990 and 2003.

Limitation: Infection was determined only by HCV seropositivity.

Conclusion: The incidence of HCC in Osaka started to decrease by 2000, mainly because of decreased HCV-related HCC.

Ann Intern Med. 2008;148:820-826. www.annals.org
For author affiliations, see end of text.

Primary liver cancer was the fifth most common cancer worldwide by 2000, with approximately 551 000 new cases recorded (1). In most countries, hepatocellular carcinoma (HCC) comprises 85% to 90% of primary liver cancer cases. With some exceptions, developed countries, including the United States, have been experiencing an increase in the incidence of primary liver cancer, considered to be due at least in part to increased prevalence of chronic hepatitis C virus (HCV) infection (2).

Japan has had one of the highest incidence rates of primary liver cancer among developed countries (age-standardized incidence rate in 1995, 25.5 per 100 000 men and 7.7 per 100 000 women) (3). Approximately 90% of liver cancer cases are HCC, which, in Japan, is mainly caused by chronic HCV infection rather than chronic hepatitis B virus infection (4). A recent report on the age-standardized incidence of primary liver cancer among Japanese men, which was calculated from 6 population-based cancer registries, showed a sharp increase that started in the mid-1970s but leveled off in the mid-1990s (5). These distinctive trends were thought to be due to the spread of HCV infection, which began in the 1920s and increased after World War II (6–8). Thus, HCV penetrated Japan earlier than Spain, Egypt, the United States, the former Soviet Union, South Africa, and Hong Kong, as evidenced by molecular clock analysis of the sequences of HCV isolates (8). However, recent temporal trends regarding incidence rates of HCC and the contribution of HCV infection have not been clearly documented in the Japanese population.

We analyzed temporal trends for HCC incidence rates between 1981 and 2003 in Osaka Prefecture (population in 2005, 8.8 million) and interpreted these in the context of HCV infection rates.

See also:

Print
Editors' Notes 821

Web-Only
Conversion of graphics into slides

METHODS

Data Collection on Incident HCC Cases

We obtained data on incident HCC cases from the Osaka Cancer Registry, which was established by the Osaka Prefectural Government in 1962. The registry collects reports on patients with newly diagnosed cancer, including demographic and cancer-related information, from all medical institutions in Osaka Prefecture (9). These have been routinely supplemented by death certificates gathered

by the Osaka Prefectural Government (9). For patients with cancer who were enrolled in the registry on the basis of their death certificate, we contacted the issuing hospital to obtain information on diagnosis and treatment and to establish the date of HCC incidence, which we determined to be the time of diagnosis at that hospital. We site-coded the data according to the International Classification of Diseases for Oncology, Third Edition (10). We included patients with HCC (codes 8170 through 8180). The protocol was approved by the ethics committee of the Osaka Medical Center for Cancer and Cardiovascular Diseases.

From 1981 to 2003, 48 166 men and 15 696 women with HCC were documented in the Osaka Cancer Registry. We calculated the annual age-standardized incidence rates of HCC (world population as a standard population) by sex between 1981 and 2003. To characterize temporal trends for HCC, we assessed 10-year, age-specific incidence rates of HCC between 1981 and 2003 in individuals age 50 to 79 years. We studied these particular age-specific rates because most HCV-related HCC cases in the Japanese population occur between the ages of 50 and 79 years (4). We used the annual population estimates from 1981 to 2003, which were based on the average population in each sex and age category for the Osaka Prefecture during the particular period, as denominators for calculating incidence rates. The annual population estimates were based on data from the 1980, 1985, 1990, 1995, 2000, and 2005 Japanese population censuses, with linear interpolation for the years in between.

Statistical Analysis

To identify years when a statistically significant change in the slope of the temporal trend in the incidence occurred, we applied the joinpoint regression model by using the Joinpoint Regression Program, version 3.0 (U.S. National Cancer Institute, Bethesda, Maryland). We assumed constant variance and uncorrelated errors (11) because we could not detect heteroskedasticity by the White test or autocorrelation by the Durbin-Watson test in men or women in any age group.

We computed the estimated slopes describing the average annual change of incidence rate per 100 000 persons and the corresponding 95% CIs for each trend by fitting a piecewise regression line to the rates, using calendar year as a regression variable. We used the permutation test method to identify years when a statistically significant change had occurred ($P < 0.05$) and set the number of randomly permuted data sets at 4499. We set the number of joinpoints to a minimum of 0 and a maximum of 3 in the Joinpoint Regression Program.

Data Collection on Prevalence of HCV Infection among Patients with HCC

The Osaka Cancer Registry does not collect serologic data on HCV infection in the registered patients. Therefore, we used data on HCV seropositivity from patients with HCC that was diagnosed at 10 hospitals in Osaka Prefecture (1 university hospital, 2 cancer centers, and 7 general hospitals) to estimate the prevalence of HCV infection in patients with HCC. We considered the HCC diagnosis confirmed when the patient had positive histologic or positive radiologic results by enhanced computed tomography or hepatic angiography. We collected data on the patient's sex, date of birth, date of diagnosis between 1990 and 2003, first Chinese letter of the family name, and presence of hepatitis B surface antigen and antibody to hepatitis C (anti-HCV) as assessed by any commercially available kit. We did not collect the full first and family name for reasons of confidentiality. Because anti-HCV testing first became available in Japan in 1990, we collected data on patients whose HCC diagnosis was between 1990 and 2003. One investigator checked for duplication of the data set, because some patients might have been registered multiple times among the participating hospitals as a result of referrals and recurrence of HCC. We defined HCV-related HCC as occurring in patients who were HCV-seropositive at the time of diagnosis.

We calculated the sex-specific, age-specific (50 to 59, 60 to 69, or 70 to 79 years), and period-specific (1990 to 1992, 1993 to 1995, 1996 to 1998, 1999 to 2001, or 2002 to 2003) prevalences of HCV seropositivity for patients with HCC. We then multiplied prevalence rates by the corresponding strata of the HCC incidence rate obtained from the Osaka Cancer Registry data. Thus, we derived the denominators from the general population in Osaka through the denominators of the HCC incidence rate and obtained the numerators by multiplying the prevalence rates by the HCC incidence rate. We calculated the incidence rate of non–HCV-related HCC by subtracting HCV-related HCC from total HCC. Thus, we describe trends for the estimated incidence rates of HCV-related

Context

Hepatitis C virus (HCV) infection in Japan began to spread during the 1920s, increased after World War II with an explosion in parenteral amphetamine use and paid blood donation, and decreased in the 1950s to 1960s with voluntary blood donation and penalties against amphetamine use. Evidence linking the trends in HCV infection to hepatocellular carcinoma rates in Japan is limited.

Contribution

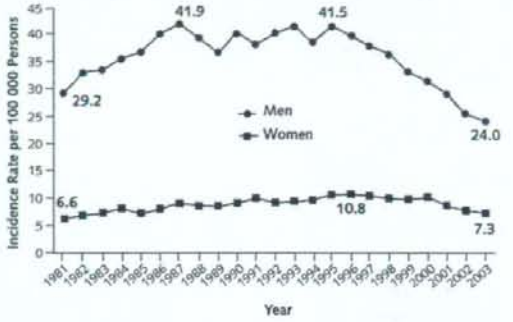
Data from the Osaka Cancer Registry and 10 Osaka hospitals suggest that hepatocellular carcinoma rates began to decrease in 2000, mainly because of a decrease in HCV-associated cancer.

Implication

Control of HCV transmission within a population seems to be followed by a decrease in hepatocellular carcinoma.

—The Editors

Figure 1. **Trends in age-standardized (world population) incidence of hepatocellular carcinoma in Osaka, Japan, 1981–2003.**



and non–HCV-related HCC between 1990 and 2003 in Osaka Prefecture. We calculated the CI of the estimated rates by multiplying the lower and upper limits of the CI of the prevalence based on SE by the corresponding HCC incidence rate.

Role of the Funding Source

This study was supported by the Osaka Prefectural Government between 1990 and 2000 and Grants-in-Aid for Hepatitis Research of the Japanese Ministry of Health, Labor, and Welfare. There is no conflict of interest in the study. The funding sources had no role in the collection, management, or analysis of data.

RESULTS

The age-standardized incidence rate of HCC in men increased between 1981 and 1987 from 29.2 to 41.9 cases per 100 000 persons, then fluctuated until 1995. After that, it steadily decreased to 24.0 cases per 100 000 persons in 2003 (**Figure 1**). Among women, the age-standardized incidence rate of HCC increased between 1981 and 1996 from 6.6 to 10.8 cases per 100 000 persons, then gradually decreased to 7.3 cases per 100 000 persons in 2003 (**Figure 1**).

Figure 2 shows the trends in the incidence of HCC among men and women age 50 to 59 years, 60 to 69 years, and 70 to 79 years in Osaka between 1981 and 2003. The HCC incidence rate increased from 1981 to 1986 among men age 50 to 59 years, from 1981 to 1995 among men age 60 to 69 years, and from 1981 to 2000 among men age 70 to 79 years (average annual change of the incidence rate [per 100 000 persons], 10.0, 10.7, and 6.2, respectively) (**Table 1**). A striking downward trend occurred after the year of peak incidence in the 3 age groups (−7.9 until 1996, −22.3 until 2003, and −12.4 until 2003, respectively). Among men age 50 to 59 years, there was a second joinpoint (a change from rapid to moderate decrease) in 1996, resulting in a slope of −3.1 until 2003. Among women age 50 to 59 years, 60 to 69 years, and 70 to 79 years, the incidence rates of HCC peaked in 1991, 1997, and 2000, respectively (**Table 1**). The rates in women seemed to increase slightly from 1981 until the year of the joinpoint, with slopes of 0.43, 2.07, and 3.10, respectively. Thereafter, HCC incidence rates in women decreased through 2003 at a statistically significant average annual rate of −0.9, −5.7, and −7.9, respectively (**Table 1**).

Figure 2. **Joinpoint analysis of the incidence rate of hepatocellular carcinoma among individuals age 50 to 79 years in Osaka, Japan, 1981–2003.**

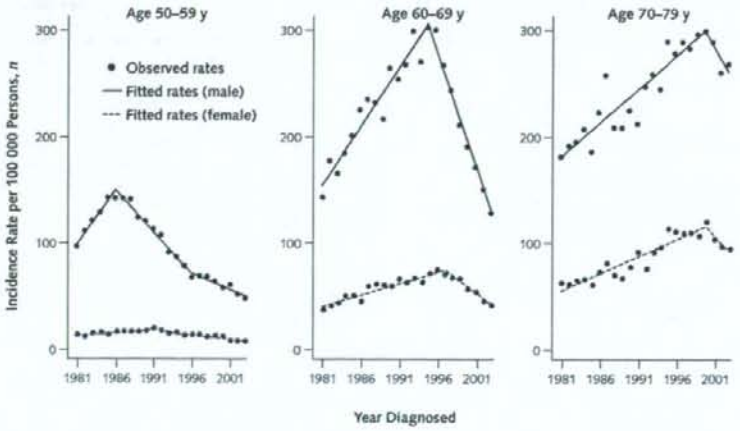


Table 1. **Joinpoint Analysis of the Hepatocellular Carcinoma Incidence Rate per 100 000 Persons in Osaka, Japan, 1981–2003**

Age Range	Peak Year	Incidence Rate per 100 000 Persons	Trend 1		Trend 2		Trend 3	
			Years	Slope (95% CI)	Years	Slope (95% CI)	Years	Slope (95% CI)
Men								
50–59 y	1986	142.0	1981–1986	10.0 (8.2 to 11.8)*	1986–1996	−7.9 (−8.6 to −7.1)*	1996–2003	−3.1 (−4.2 to −2.1)*
60–69 y	1995	299.6	1981–1995	10.7 (9.1 to 12.3)*	1995–2003	−22.3 (−26.0 to −18.6)*	–	–
70–79 y	2000	296.4	1981–2000	6.2 (4.8 to 7.5)*	2000–2003	−12.4 (−35.7 to 10.9)	–	–
Women								
50–59 y	1991	19.7	1981–1991	0.4 (0.2 to 0.7)*	1991–2003	−0.9 (−1.1 to −0.7)*	–	–
60–69 y	1997	68.5	1981–1997	2.1 (1.7 to 2.4)*	1997–2003	−5.7 (−7.3 to −4.1)*	–	–
70–79 y	2000	118.1	1981–2000	3.1 (2.5 to 3.7)*	2000–2003	−7.9 (−18.1 to 2.4)	–	–

* $P < 0.001$.

Table 2 shows the prevalence of anti-HCV antibodies among 5253 patients age 50 to 79 years with HCC that was diagnosed at 10 hospitals in Osaka between 1990 and 2003. The prevalence was highest in men with HCC that was diagnosed in 1993 to 1995 (82.4%). The proportion of HCV-seronegative patients ranged from 18% to 29% through the observation period. The prevalence of anti-HCV was almost constant (81% to 83%) among women with HCC that was diagnosed between 1993 and 2003 (**Table 2**).

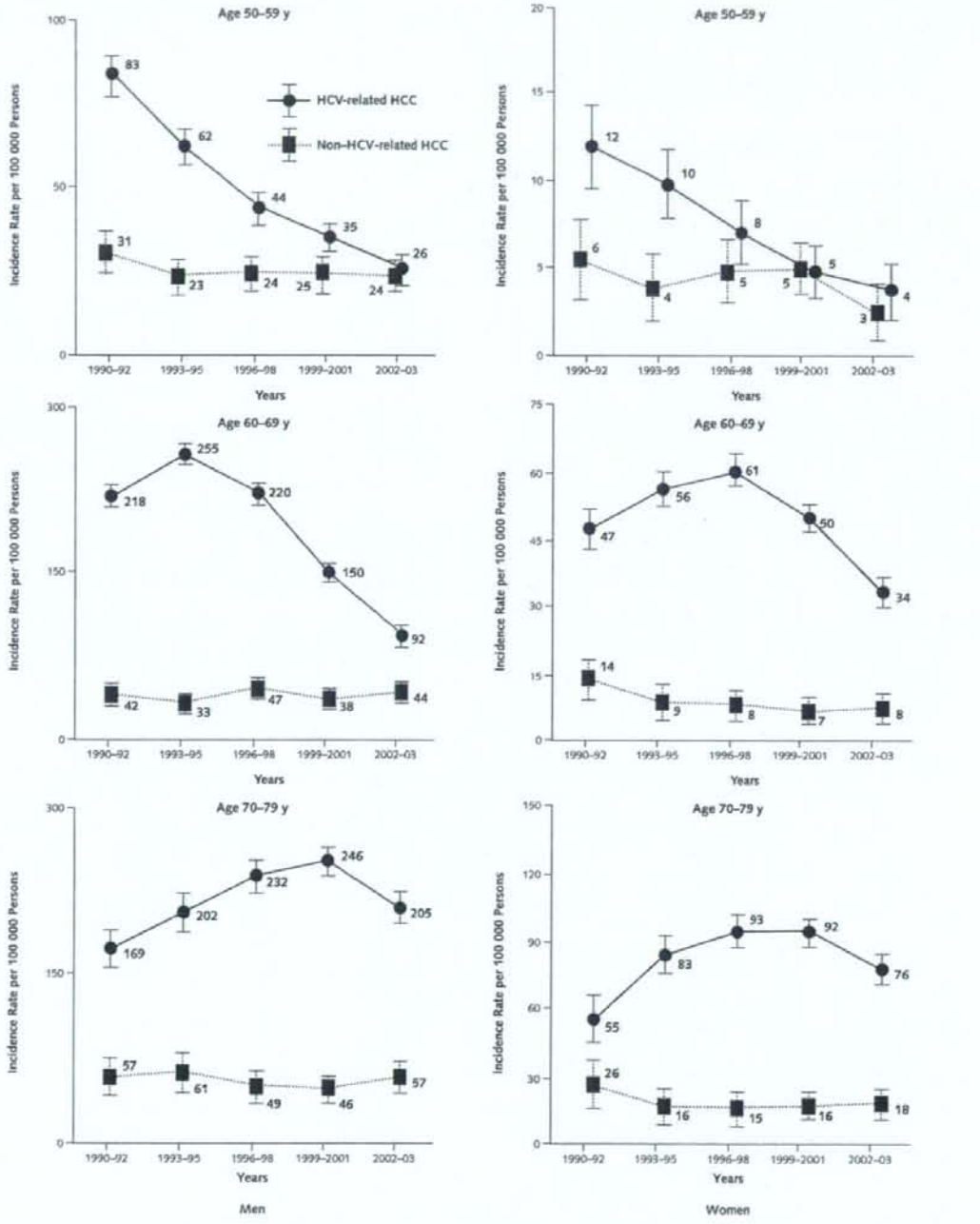
Figure 3 shows changes in the estimated incidence rate of HCV-related and non–HCV-related HCC from 1990 to 2003. Among men, the estimated incidence rate of HCV-related HCC steadily decreased among Osaka residents age 50 to 59 years from 83 (95% CI, 77 to 89) cases per 100 000 persons in 1990 to 1992 to 26 (CI, 21 to 30) cases per 100 000 persons in 2002 to 2003. Among men age 60 to 69 years, incidence seemed to peak (255 [CI, 247 to 264] cases per 100 000 persons) from 1993 to 1995. Among men age 70 to 79 years, the incidence rate increased from 1990 to 1992 (169 [CI, 153 to 186] cases per 100 000 persons) to 1999 to 2001 (246 [CI, 234 to 258] cases per 100 000 persons) and leveled off afterward. The estimated incidence rate of HCV-related HCC among women age 50 to 59 years decreased from 12.4 (CI, 10.1 to 14.7) cases per 100 000 persons during 1990 to 1992 to 4.2 (CI, 2.5 to 5.8) cases per 100 000 persons during 2002 to 2003, whereas among women age 60 to 69 years, the incidence peaked (61 [CI, 57 to 64] cases per 100 000 persons) during 1996 to 1998. The trend in women age 70 to 79 years seemed to be similar to that in men of the same age: increasing during the 1990s and leveling off in the early 2000s (**Figure 3**). The estimated incidence rate of non–HCV-related HCC was lower than that of HCV-

Table 2. **Prevalence of Anti-HCV among 5253 Patients Age 50 to 79 Years with Hepatocellular Carcinoma at 10 Hospitals in Osaka, Japan, 1990–2003***

Variable	1990–1992		1993–1995		1996–1998		1999–2001		2002–2003	
	Patients, *n*	Prevalence (±SE), %	Patients, *n*	Prevalence (±SE), %	Patients, *n*	Prevalence (±SE), %	Patients, *n*	Prevalence (±SE), %	Patients, *n*	Prevalence (±SE), %
Men										
Anti-HCV(+)	602	78.3 ± 1.5	677	82.4 ± 1.3	651	78.7 ± 1.4	709	76.6 ± 1.4	385	70.9 ± 1.9
Anti-HCV(+) and HBsAg(+)	18	2.3 ± 0.5	17	2.1 ± 0.5	11	1.3 ± 0.4	16	1.7 ± 0.4	8	1.5 ± 0.5
Anti-HCV(+) and HBsAg(−)	584	75.9 ± 1.5	660	80.3 ± 1.4	640	77.4 ± 1.5	693	74.8 ± 1.4	377	69.4 ± 2.0
Anti-HCV(−)	167	21.7 ± 1.5	145	17.6 ± 1.3	176	21.3 ± 1.4	217	23.4 ± 1.4	158	29.1 ± 1.9
Anti-HCV(−) and HBsAg(+)	60	7.8 ± 1.0	57	6.9 ± 0.9	71	8.6 ± 1.0	106	11.4 ± 1.0	68	12.5 ± 1.4
Anti-HCV(−) and HBsAg(−)	107	13.9 ± 1.2	88	10.7 ± 1.1	105	12.7 ± 1.2	111	12.0 ± 1.1	90	16.6 ± 1.6
Total	769	100.0	822	100.0	827	100.0	926	100.0	543	100.0
Women										
Anti-HCV(+)	165	73.0 ± 3.0	211	82.7 ± 2.4	248	82.9 ± 2.2	274	80.8 ± 2.1	200	81.0 ± 2.5
Anti-HCV(+) and HBsAg(+)	8	3.5 ± 1.2	2	0.8 ± 0.6	5	1.7 ± 0.7	2	0.6 ± 0.4	2	0.8 ± 0.6
Anti-HCV(+) and HBsAg(−)	157	69.5 ± 3.1	209	82.0 ± 2.4	243	81.3 ± 2.3	272	80.2 ± 2.2	198	80.2 ± 2.5
Anti-HCV(−)	61	27.0 ± 3.0	44	17.3 ± 2.4	51	17.1 ± 2.2	65	19.2 ± 2.1	47	19.0 ± 2.5
Anti-HCV(−) and HBsAg(+)	21	9.3 ± 1.9	17	6.7 ± 1.6	29	9.7 ± 1.7	29	8.6 ± 1.5	18	7.3 ± 1.7
Anti-HCV(−) and HBsAg(−)	40	17.7 ± 2.5	27	10.6 ± 1.9	22	7.4 ± 1.5	36	10.6 ± 1.7	29	11.7 ± 2.0
Total	226	100.0	255	100.0	299	100.0	339	100.0	247	100.0

* HBsAg = hepatitis B surface antigen; HCV = hepatitis C virus.

Figure 3. Trends in estimated incidence rates of hepatitis C virus (*HCV*)–related and non–HCV-related hepatocellular carcinoma (*HCC*) in Osaka, Japan, 1990–2003.



Information on anti-HCV status only became available after 1989. Error bars indicate 95% CIs.

related HCC in most strata. We observed no distinctive changes in the temporal trends for non–HCV-related HCC during the study period.

Discussion

Our analysis of HCC incidence in the Japanese population between 1981 and 2003 identified calendar years in which significant changes in temporal trends occurred. The HCC incidence rates in men and women age 50 to 59 years peaked during 1986 and 1991, respectively; in men and women age 60 to 69 years during 1995 and 1997, respectively; and in men and women age 70 to 79 years in 2000. We also found that temporal trends for HCC incidence between 1990 and 2003 by age group were mainly determined by trends in the incidence rates of HCV-related HCC.

The most likely explanation for these observations is the particular mode of HCV transmission in Japanese society. According to a study on molecular tracing of endemic HCV (8), the exponential spread of HCV-1b infection, a dominant genotype of HCV in Japan, started in the 1920s. This was associated with treatment of *Schistosoma japonicum* beginning in 1921 (12). Later, HCV infection coincided with an increase in parenteral amphetamine use in the devastated country during and after World War II (6, 7). Subsequently, viral spread was considered to be amplified through blood transfusions and parenteral medical procedures in the 1950s and 1960s (6, 7). Data on first-time blood donor candidates in Osaka indicate that the prevalence of anti-HCV antibodies among those born in 1925 to 1935 was much higher (7% to 10%) than that in the younger generation born in 1936 to 1955 (13). It is plausible that Japanese people born between 1925 and 1935, who were adolescents in the early 1950s, were most susceptible to HCV transmission under these circumstances. Age groups with peak incidence of HCC in men and women in the current study (1986 and 1991, respectively, for 50 to 59 years; 1995 and 1997, respectively, for 60 to 69 years; and 2000 for 70 to 79 years) included the generation for which prevalence of anti-HCV was high in Osaka (born in 1925 and 1935) (13). Stiffening of legal penalties against amphetamine use starting in 1954 and conversion from paid to voluntary blood donation in the late 1960s may have reduced HCV transmission, thereby resulting in the lower prevalence of HCV infection in generations born after 1935. Indeed, the spread of HCV in Japan essentially ended by the early 1990s at the latest, as evidenced by the current very low incidence of HCV infection among repeat blood donors (14, 15). Better detection methods introduced in the early 1980s for HCC in patients with cirrhosis through ultrasonography and measurement of α-fetoprotein may have contributed to the apparent increase in the incidence of HCC found in this study. However, the distinctive changes we observed in the age-specific incidence of HCC during the 1990s through the early 2000s cannot be explained by the increased ability to detect HCC, because the different joinpoints in age-specific incidence rates would not be derived from a single period effect of detection of HCC.

Increases in the incidence of and deaths from liver cancer in the 1970s to 1990s have been reported in Japan (5, 16), Australia (2), the United Kingdom (17), France (2, 18), Italy (2, 18), and the United States (2, 19). The increases in Japan and the United States are attributable to increased seroprevalence of HCV (6, 13, 20, 21), whereas this relationship has not been clearly established in the other countries.

Certain limitations of this study should be considered. First, because cancer reporting in Osaka is not mandated by law, HCC could have been underreported. However, because it is fatal, most of the unreported cases should have been detected by examination of the death certificate. In addition, because the proportion of persons with HCC included only on the basis of their death certificate was almost constant (22% to 25%) during the observation period (22–24), such underreporting would not be expected to affect the temporal trends for HCC incidence rates shown in our study. Second, the proportion of HCV-seropositive patients among the 5253 cases diagnosed at 10 hospitals might differ somewhat from the entire cohort of patients with HCC in Osaka. However, all Japanese patients, including those with HCC, have easy access to hospitals because of the national medical insurance system, and the 10 participating hospitals did not select patients with HCC on the basis of their etiologic background. Therefore, it is realistic to suppose that selection bias on prevalence of anti-HCV among these 5253 patients would have been limited. Finally, the temporal trends seen in the present study might differ from those among the entire Japanese population. We previously reported age-specific incidence rates of liver cancer by birth year in Japanese men between 1962 and 1997 (5) by using 6 population-based cancer registries from Cancer Incidence in Five Continents (9) (registries for Miyagi, Yamagata, Osaka, Hiroshima, Saga, and Nagasaki). Our previous study found the peak incidence of HCC among those born between 1931 and 1935 (5). In addition, the age-dependent prevalence of anti-HCV among first-time blood donors in Osaka (13) was similar to those in other areas of Japan (25). These findings may indicate that the timing of the outbreak of HCV infection and its reduction were similar in the different geographic areas of the country.

In conclusion, our calculation of HCC incidence rates demonstrated that they are already decreasing in both sexes in Osaka, Japan. That the outbreak of HCV infection in Japan after World War II and its termination occurred earlier in Japan than in the rest of the world is the most likely explanation for these observations. These findings confirm that HCV-related HCC is a preventable disease that can be decreased by controlling parenteral HCV transmission. In the early 1990s, interferon therapy for patients

with chronic HCV infection was started in Japan to reduce the risk for HCC (26, 27). A nationwide, community-based anti-HCV screening system targeting individuals age 40 to 70 years was introduced by municipal governments in Japan in 2002. Further observation of the temporal trends of HCC incidence is needed to assess the efficacy of these interventions in Japan.

From Osaka Medical Center for Cancer and Cardiovascular Diseases, Ikeda Municipal Hospital, Osaka University Graduate School of Medicine, Osaka Police Hospital, National Hospital Organization Osaka Minami Medical Center, Osaka Koseinenkin Hospital, Otemae Hospital, Osaka Rousai Hospital, Osaka General Medical Center, and National Hospital Organization Osaka National Hospital, Osaka, Japan.

Acknowledgment: The authors thank the Osaka Cancer Registry for allowing use of their data and Ms. Yasue Kotani for assistance with statistical analysis.

Grant Support: By the Osaka Prefectural Government (1999–2000) and Grants-in-Aid for Hepatitis Research of the Japanese Ministry of Health, Labor, and Welfare.

Potential Financial Conflicts of Interest: None disclosed.

Reproducible Research Statement: *Study protocol:* Available by contacting Dr. Tanaka (e-mail, hitanaka@aichi-cc.jp). The protocol is only available in Japanese. *Statistical code and data set:* Not available.

Requests for Single Reprints: Hideo Tanaka, MD, 1-1 Kanokoden, Chikusa-ku Nagoya-shi, Aichi, Japan 464-8681; e-mail, hitanaka@aichi-cc.jp.

Current author addresses and author contributions are available at www.annals.org.

References

1. **Ferlay J, Bray F, Pisani P, Parkin DM, Ferlay J.** GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide, Version 1.0. IARC Cancer Base No. 5. Lyon, France: International Agency for Research on Cancer; 2001.

2. **McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni JF Jr.** International trends and patterns of primary liver cancer. Int J Cancer. 2001;94:290-6. [PMID: 11668511]

3. **The Research Group for Population-based Cancer Registration in Japan.** Cancer incidence and incidence rates in Japan in 1995: estimates based on data from nine population-based cancer registries. Jpn J Clin Oncol. 2000;30:318-21. [PMID: 11007166]

4. **Tanaka H, Tsukuma H.** Hepatitis C virus. In: Newton R, Beral V, Weiss RA, Tooze J, eds. Cancer Surveys Vol. 33: Infections and Human Cancer. New York: Cold Spring Harbor Laboratory Pr; 1999:213-35.

5. **Tanaka H, Uera F, Tsukuma H, Ioka A, Oshima A.** Distinctive change in male liver cancer incidence rate between the 1970s and 1990s in Japan: comparison with Japanese-Americans and US whites. Jpn J Clin Oncol. 2007;37:193-6. [PMID: 17332055]

6. **Yoshizawa H.** Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology. 2002;62 Suppl 1:8-17. [PMID: 11868791]

7. **Tsukuma H, Tanaka H, Ajiki W, Oshima A.** Liver cancer and its prevention. Asian Pac J Cancer Prev. 2005;6:244-50. [PMID: 16235981]

8. **Tanaka Y, Kurbanov F, Mano S, Orito E, Vargas V, Esteban JI, et al.** Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. Gastroenterology. 2006;130:703-14. [PMID: 16530512]

9. **Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DM, eds.** Cancer Incidence in Five Continents. vol. VIII. IARC Scientific Publications No. 155. Lyon, France: International Agency for Research on Cancer; 2002:264-5.

10. **Fritz A, Percy C, Jack A, Shanmugaratnam K, Sobin L, Parkin DM, Whelans, eds.** The International Classification of Diseases for Oncology, Third Edition. Geneva: World Health Organization; 2000.

11. **Kim HJ, Fay MP, Feuer EJ, Midthune DN.** Permutation tests for joinpoint regression with applications to cancer rates. Stat Med. 2000;19:335-51. [PMID: 10649300]

12. **Iida F, Iida R, Kamijo H, Takaso K, Miyazaki Y, Funabashi W, et al.** Chronic Japanese schistosomiasis and hepatocellular carcinoma: ten years of follow-up in Yamanashi Prefecture, Japan. Bull World Health Organ. 1999;77:573-81. [PMID: 10444881]

13. **Tanaka H, Hiyama T, Tsukuma H, Okubo Y, Yamano H, Kitada A, et al.** Prevalence of second generation antibody to hepatitis C virus among voluntary blood donors in Osaka, Japan. Cancer Causes Control. 1994;5:409-13. [PMID: 7999962]

14. **Sasaki F, Tanaka J, Moriya T, Katayama K, Hiraoka M, Ohishi K, et al.** Very low incidence rates of community-acquired hepatitis C virus infection in company employees, long-term inpatients, and blood donors in Japan. J Epidemiol. 1996;6:198-203. [PMID: 9002386]

15. **Tanaka H, Tsukuma H, Hori Y, Nakade T, Yamano H, Kinoshita N, et al.** The risk of hepatitis C virus infection among blood donors in Osaka, Japan. J Epidemiol. 1998;8:292-6. [PMID: 9884479]

16. **Tanaka H, Tsukuma H.** Characteristics of Japanese patients with liver cancer—epidemiological study based on a comparison between male and female patients. Hepatol Res. 2002;24:S11-20.

17. **Taylor-Robinson SD, Foster GR, Arora S, Hargreaves S, Thomas HC.** Increase in primary liver cancer in the UK, 1979-94 [Letter]. Lancet. 1997;350:1142-3. [PMID: 9343506]

18. **La Vecchia C, Lucchini F, Franceschi S, Negri E, Levi F.** Trends in mortality from primary liver cancer in Europe. Eur J Cancer. 2000;36:909-15. [PMID: 10785597]

19. **El-Serag HB, Davila JA, Petersen NJ, McGlynn KA.** The continuing increase in the incidence of hepatocellular carcinoma in the United States: an update. Ann Intern Med. 2003;139:817-23. [PMID: 14623619]

20. **El-Serag HB, Mason AC.** Risk factors for the rising rates of primary liver cancer in the United States. Arch Intern Med. 2000;160:3227-30. [PMID: 11088082]

21. **Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB.** Hepatitis C infection and the increasing incidence of hepatocellular carcinoma: a population-based study. Gastroenterology. 2004;127:1372-80. [PMID: 15521006]

22. **Parkin DM, Muir CS.** Comparability and quality of data. In: Parkin DM, Muir CS, Whelan SL, Gao YT, Ferlay J, Powell J, eds. Cancer Incidence in Five Continents. vol. VI. IARC Scientific Publications No. 120. Lyon, France: International Agency for Reseach on Cancer; 1992:45-173.

23. Indices of data quality. In: Parkin DM, Whelan SL, Ferlay J, Raymond L, Young J, eds. Cancer Incidence in Five Continents. vol. VII. IARC Scientific Publications No. 143. Lyon, France: International Agency for Research on Cancer; 1997:1146.

24. Indices of data quality. In: Parkin DM, Whelan SL, Ferlay J, Raymond L, Teppo L Thomas DB, eds. Cancer Incidence in Five Continents. vol. VII. IARC Scientific Publications No. 143. Lyon, France: International Agency for Research on Cancer; 2002:719.

25. **Yoshizawa H.** Trends of hepatitis virus carriers. Hepatology Research 2002; 24: S28-39.

26. **Imai Y, Kawata S, Tamura S, Yabuuchi I, Noda S, Inada M, et al.** Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. Ann Intern Med. 1998;129:94-9. [PMID: 9669992]

27. **Tanaka H, Tsukuma H, Kasahara A, Hayashi N, Yoshihara H, Masuzawa M, et al.** Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: a retrospective cohort study of 738 patients. Int J Cancer. 2000;87:741-9. [PMID: 10925370]

Current Author Addresses: Drs. Tanaka, Ito, Imanaka, and Tsukuma: 1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan.
Dr. Imai: 3-1-18 Johnan, Ikeda, Osaka 563-0025, Japan.
Drs. Hiramatsu and Takehara: 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
Dr. Oshita: 10-31 Kitayama-cho, Tennouji-ku, Osaka 543-0035, Japan.
Dr. Hijioka: 2-1 Kido-Higashi-cho, Kawashinagano, Osaka 586-0008, Japan.
Dr. Katayama: 4-2-78 Fukushima, Fukushima-ku, Osaka 553-0003, Japan.
Dr. Yabuuchi: 1-5-34 Otemae, Chuo-ku, Osaka 540-0008, Japan.
Dr. Yoshihara: 1179-3 Nagasone-cho, Kita-ku, Sakai, Osaka 591-8025, Japan.
Dr. Inoue: 3-1-56 Bandai-Higashi, Sumiyoshi-ku, Osaka 558-0056, Japan.
Dr. Kato: 2-1-14 Hoenzaka, Chuo-ku, Osaka 540-0006, Japan.
Dr. Tamura: 5-7-1 Kayano, Minoh, Osaka 562-0014, Japan.
Drs. Kasahara and Hayashi: 2-15 Yamadaoka, Suita, Osaka 565-0871, Japan.

Author Contributions: Concept and design: H. Tanaka, Y. Imai, N. Hiramatsu.
Analysis and interpretation of the data: Y. Imai, K. Imanaka, M. Oshita, T. Hijioka, K. Katayama, I. Yabuuchi, H. Yoshihara, A. Inoue, M. Kato, T. Takehara, S. Tamura, A. Kasahara, H. Tsukuma.
Drafting of the article: H. Tanaka.
Final approval of the article: H. Tanaka, Y. Imai, N. Hiramatsu, Y. Ito, K. Imanaka, M. Oshita, T. Hijioka, K. Katayama, I. Yabuuchi, H. Yoshihara, A. Inoue, M. Kato, T. Takehara, S. Tamura, A. Kasahara, N. Hayashi, H. Tsukuma.
Statistical expertise: Y. Ito.

Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

Keisuke Kohga,[1,5] Tetsuo Takehara,[1,5] Tomohide Tatsumi,[1] Kazuyoshi Ohkawa,[1] Takuya Miyagi,[1] Naoki Hiramatsu,[1] Tatsuya Kanto,[1] Tsutomu Kasugai,[2] Kazuhiro Katayama,[2] Michio Kato[3] and Norio Hayashi[1,4]

[1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; [2]Department of Gastroenterology and Hepatology, Osaka Koseinenkin Hospital, 4-2-27 Fukushima, Fukushimaku, Osaka, Osaka 553-0003; [3]Department of Gastroenterology and Hepatology, National Hospital Organization, Osaka National Hospital, 2-1-14 Hoenzaka, Chuoku, Osaka, Osaka 540-0006, Japan

(Received January 16, 2008/Revised April 8, 2008/Accepted April 11, 2008/Online publication July 29, 2008)

Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial emboli-zation therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

MHC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.[1] In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.[2–5] The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.[6] Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.[7] In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.[8–10] The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.[4] In addition, sera from those patients can downregulate NKG2D expression *in vitro*.[5,11] These data suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.[12,13]

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.[14,15] With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.[16] We reported previously that soluble MICA could be detected in sera of HCC patients.[17] However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

*To whom correspondence should be addressed.
E-mail: hayashin@gh.med.osaka-u.ac.jp
[5]Keisuke Kohga and Tetsuo Takehara contributed equally to this work.
Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

doi: 10.1111/j.1349-7006.2008.00859.x

Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH		0/5/	2/1/	4/0/
AIH/PBC/others		2/0/0	1/6/4	0/0/3
Child–Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.
*$P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2-test; **$P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; ***$P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; ****$P < 0.05$ vs cirrhosis by χ^2-test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.[18] Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child–Pugh classification, and TNM staging of HCC. Child–Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.[16]

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparafinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the α1 and α2 domains of MIC molecules shared by both MICA and MICB.[2] To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child–Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child–Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal–Wallis rank sum test was used. If the Kruskal–Wallis test was significant, post hoc multiple comparisons were carried out using the Steel–Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t-test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

doi: 10.1111/j.1349-7006.2008.00859.x

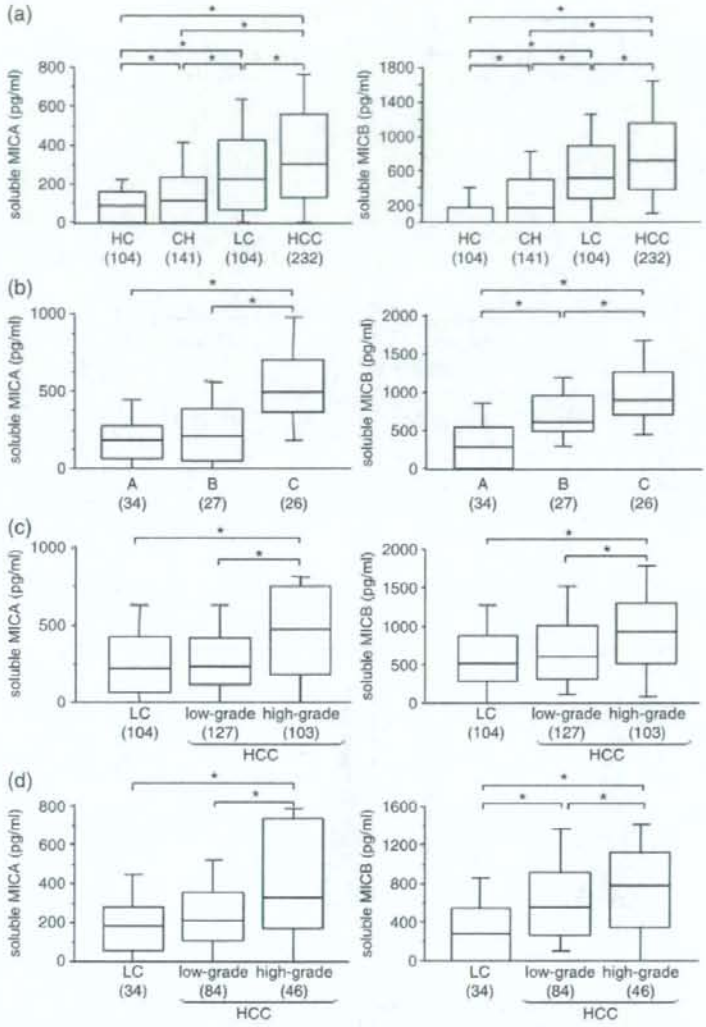


Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child–Pugh classification. (c,d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child–Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. $*P < 0.05$ by Kruskal–Wallis test and post hoc Steel–Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhosis patients were stratified based on Child–Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child–Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a