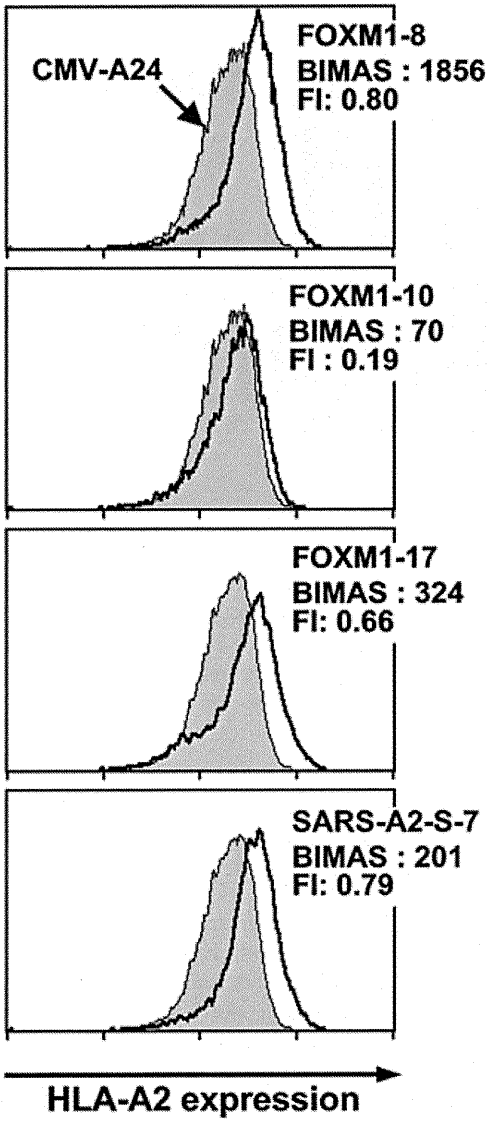

Figure 6. ›Binding of the FOXM1-derived peptides to HLA-A2. Three FOXM-1 derived peptides were examined on the actual binding to HLA-A2 by using a TAP-deficient, *HLA-A*0201*-positive cell line T2. T2 cells, pre-incubated overnight at 26°C, were cultured in the presence of 50 μM peptide and 5 μg/ml β_2-microglobulin in serum-free medium at 26°C for 1.5 hr and subsequently at 37°C for 18 hr. After the culture, HLA-A2 expression on the cell surface was measured by flow cytometry with anti-HLA-A2 monoclonal antibody BB7.2. A human cytomegalovirus-derived peptide (QYDPVAALF) and a SARS-A2-S-7 peptide (NLNESLIDL) were used as a negative and a positive control, respectively. The fluorescence index (FI) was calculated from the mean fluorescence intensity (MFI) of HLA-A2 expressed on T2 cells determined by flow cytometry, using the formula FI = (MFI [T2 cells with FOXM1 peptide]/MFI [T2 cells with CMV peptide]) − 1. BIMAS score predicting HLA-A2-binding affinity of the peptides are also indicated.

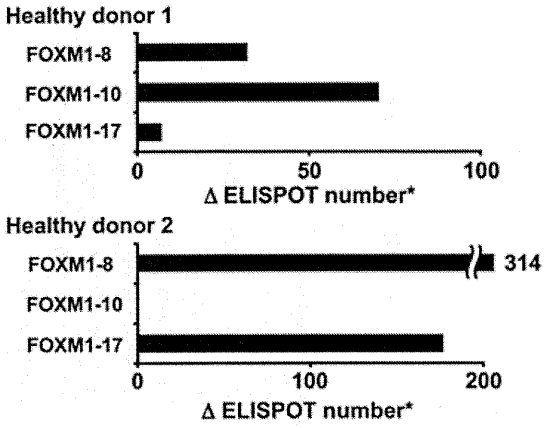

Figure 7. CTL induction from PBMCs of HLA-A2-positive healthy donors. FOXM1 peptide-reactive CTLs were generated from $CD8^+$ T cells of $HLA\text{-}A2^+$ healthy donors. After 3 or 4 times of stimuli with autologous monocyte-derived DCs pulsed with the FOXM1-8, FOXM1-10, or FOXM1-17 peptide, the CTLs were subjected to IFN-γ ELISPOT assay, using T2 cells pulsed with the same FOXM1 peptide as stimulators. The number of specific spots was determined by subtracting the mean number of spots in the absence of an antigen from that in the presence of the antigen: (ELISPOT number stimulated with T2 cells + peptide) − (ELISPOT number stimulated with T2 cells alone).

Thereafter, we investigated human CTL response to FOXM1. We tried to establish FOXM1-reactive CTL lines from PBMCs of HLA-A2-positive healthy individuals by stimulation with the FOXM1-8, FOXM1-10 or FOXM1-17 peptides. $CD8^+$ T cells sorted from the PBMCs were co-cultured with autologous monocyte-derived DCs pulsed with the peptides as described in the material and methods. CTL lines generated from HLA-A2 positive healthy donors produced IFN-γ specifically in response to re-stimulation with T2 cells pulsed with the peptides (Fig. 7).

Further investigation examined whether the identified peptides were produced from FOXM1 protein by intracellular natural processing. FOXM1-specific killing activity of CTLs induced with these peptides from HLA-A2-positive cancer patients were analyzed by ^{51}Cr release assay. RT-PCR analyses revealed that all of the HLA-A2 positive cell lines that we examined expressed *FOXM1* (Fig. 2, Supporting Information Fig. 1, and data not shown), except for Caco-2 cells. Thus, we introduced Caco-2 cells with an expression vector for *FOXM1* by using lentivirus and used as target cells (Caco-2-FOXM1). Caco-2 cells infected with lentiviruses without *FOXM1*-gene were also used as negative control target cells (Caco-2-mock). The expression of transgene-derived protein in the transfectants was confirmed as shown in Figure 8*a*. The peptide-induced CTL lines exhibited significantly higher cytotoxicity against Caco-2-FOXM1 cells than against Caco-

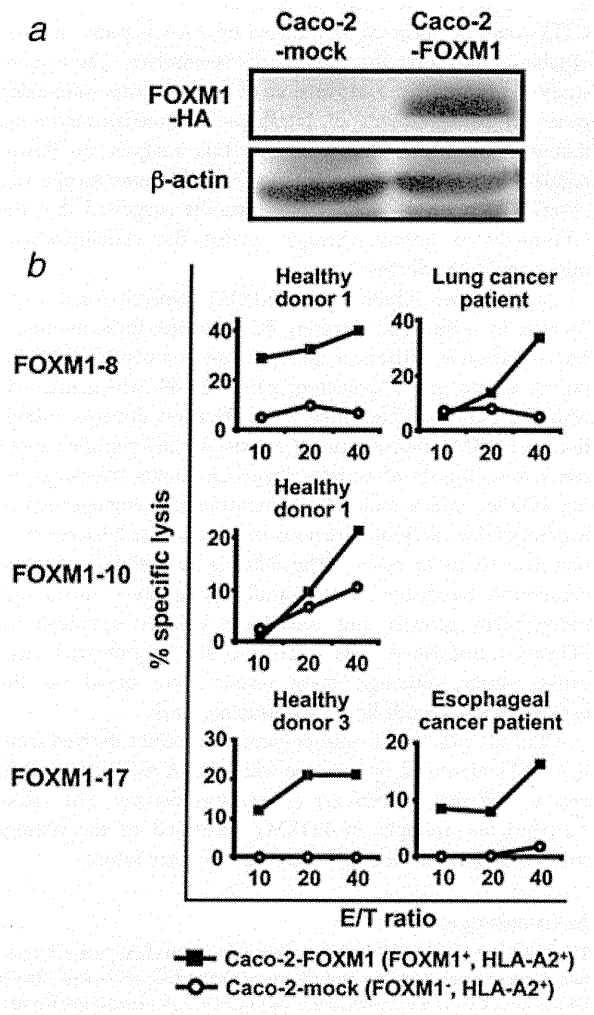

Figure 8. Natural processing of FOXM1 peptides. (*a*) Total cell lysate from Caco-2 cells infected with Lentiviruses without *FOXM1*-gene (Caco-2-mock) or those introduced with HA-tagged human *FOXM1*-gene (Caco-2-FOXM1) was analyzed by Western blot analysis for transgene-derived FOXM1expression, using anti-HA antibody. As a loading control, the same membrane was blotted with an anti-β-actin antibody. (*b*) Cytotoxic activity of the CTL lines established from *HLA-A*0201*-positive healthy donors and patients with cancer by stimulation with the 3 FOXM1-derived peptides against Caco-2-mock and Caco-2-FOXM1 (FOXM1$^+$, *HLA-A*0201*$^+$) was examined by ^{51}Cr release assay. The percentage of specific lysis was calculated from the mean values of duplicate or triplicate assays.

2-mock cells. These results clearly indicate that these peptides were naturally processed from FOXM1 protein and presented in the context of HLA-A2 to be recognized by peptide-induced CTLs.

Discussion

The identification of adequate target antigens which induce a potent and specific anti-tumor immune response is important for the development of cancer immunotherapy. Although many TAAs in various cancers have been identified,[32,33] only a few antigens specifically expressed in cholangiocarcinoma have been reported[34,35] A previous cDNA microarray analysis identified multiple gene transcripts that are up- or down-regulated in cholangiocarcinoma.[15] The current study searched for TAA by using the cDNA microarray data and identified FOXM1 as a candidate for target antigen.

FOXM1 is over-expressed in a number of aggressive human carcinomas.[12,15–21] An enhanced expression of FOXM1 was also observed in various cancer tissues in cDNA microarray analysis (Supporting Information Table 1). The expression of FOXM1 is significantly associated with poor overall survival of non-small cell lung cancer and glioblastoma patients.[36,37] Therefore, FOXM1 may have the potential to serve as a good target of cancer immunotherapy for various types of cancers. To evaluate FOXM1 as a target for cancer immunotherapy, the present study examined the immunogenicity of FOXM1 in human immune system.

The FOXM1-derived peptides predicted to bind to HLA-A2 were analyzed for their capacity to induce CTLs. Stimulation of PBMCs obtained from healthy donors with FOXM1-8 (YLVPIQFPV), FOXM1-10 (SLVLQPSVKV) and FOXM1-17 (GLMDLSTTPL) peptides resulted in the generation of peptide-reactive CTL lines. Furthermore, CTLs reactive to the peptides generated from cancer patients exhibited specific cytotoxicity against *HLA-A*0201*-positive Caco-2 cells introduced with an expression vector for FOXM1, but not against mock-introduced Caco-2 cells, thus verifying the natural processing of these peptides from FOXM1 protein in cancer cells. These results suggest that FOXM1 is a promising target for various types of cancers, and that the 3 peptides derived from FOXM1 are effective tools for the clinical application of peptide-based immunotherapy for *HLA-A*0201*-positive cancer patients.

The human *FOXM1* gene has a 10-exon structure. Two exons, Va and VIIa, are alternatively spliced.[38] Differential splicing of exons Va and VIIa in human *FOXM1* yields 3 isoforms, *FOXM1a*, *FOXM1b* and *FOXM1c*. *FOXM1a* contains both alternative exons, *FOXM1b* contains none of the alternative exons and *FOXM1c* only retains exon Va.[23] *FOXM1b* and *FOXM1c* exhibit transactivating activity because of the absence of VIIa, which is the inhibitory sequence. The presence of VIIa in *FOXM1a* renders it transcriptionally inactive.[23] In the present study, *FOXM1* was found to be expressed in various cancer cell samples, with a differential pattern of expression in normal testis and thymus. The *FOXM1c* isoform was more abundant in cultured cells, including those from the normal gastrointestinal tract and these results were consistent with the findings of other studies.[39,40]

FOXM1, a transcription factor, plays an important role in regulating the expression of genes involved in cell growth, proliferation, differentiation, longevity and transformation. Expression profiling has identified a *FOXM1*-regulated cluster of genes, including many G2/M-specific genes (Cyclin B, CENP-F, Nek-2, *etc*).[19,41] The loss of *FOXM1* expression in cancer cell lines results in mitotic spindle defects, delays cells in mitosis, and induces mitotic catastrophe.[19,41] These findings suggest that *FOXM1* expression is essential for cancer cell growth and survival. Indeed, evident expression of *FOXM1* mRNA was detected in most of the cell lines examined by RT-PCR in the present study. If a molecule expressed by cancer cells plays an essential role in their survival, then cancer cells cannot escape from the immune cell attack directed to this molecule by losing expression of this molecule.[42] In this regard, FOXM1 is thus considered to be a suitable target for anti-cancer immunotherapy.

HLA Tgm is not only useful for the identification of HLA-restricted antigenic epitopes but also for the evaluation of autoimmunity caused by immunization of peptides that are conserved between human and mouse FOXM1. The amino acid sequences of the FOXM1-8 and FOXM1-10 peptides are conserved between human and mouse. In the present study, vaccination with these peptides did not induce autoimmunity in HLA-A2 Tgm. These results suggest that the use of these peptides for the *HLA-A*0201*-positive humans would be safe, although low levels of expression of FOXM1 was detected in some normal organs (Figs. 1 and 3).

A number of clinical trials have evaluated peptides derived from TAAs. However, tumor regression has not so far been observed as expected.[43] The possible reasons for the lack of clinical efficacy could be an entry of advanced cancer patients in immunotherapy trials, and loss or down-regulation of HLA class I molecules, co-stimulatory molecules, and proteins associated with antigen presentation (such as TAP, low-molecular-weight protein (LMP) and β_2-microglobulin) in the tumor cells.[44] Consequently, a significant portion of tumor cells could escape from CTLs specific to the TAA-derived peptides presented by HLA class I molecules, even if CTLs could be successfully induced by TAA peptide vaccines capable of targeting the tumor cells themselves. The present study investigated the expression of tumor escape-associated genes in 25 specimens of intrahepatic cholangiocarcinoma tissues using cDNA microarray. In this analysis, no down-regulation of these genes associated with immune escape was observed (data not shown). These results suggested that the CTL-mediated immunotherapy against the cholangiocarcinoma could be effective.

Recently, two inhibitors for FOXM1 transcriptional activity were identified and targeting FOXM1 with these inhibitors was an effective anticancer therapy. One is a peptide containing an amino acid sequence of $p19^{ARF}$24-49, which interacts with the FOXM1 C-terminal transactivation domain; inhibition of FOXM1 transcriptional activity by the peptide caused selective apoptosis of hepatocellular carcinoma over-expressing FOXM1 and a reduced proliferation and angiogenesis in hepatocellular carcinoma regions but not in the adjacent normal liver tissue in mice.[45] The other is an antibiotic thiazole compound Siomycin A; this antibiotic inhibits anchorage-independent growth and selectively induces apoptosis in FOXM1-transformed cells.[46] They could be potential anti-cancer drugs, although these results were based on the experiments with cell lines or transgenic mice.

Clinical trials of the epitope-peptide vaccines derived from the TAAs identified by genome-wide cDNA microarray analyses in different cancers are currently underway. The HLA-A2-restricted epitopes of FOXM1 identified in the present study may well be clinically tested in the near future.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Transcatheter arterial infusion chemotherapy with cisplatin–lipiodol suspension in patients with hepatocellular carcinoma

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Abstract
Purpose The aim of this study was to investigate the antitumor efficacy of treatment, identify prognostic factors, and construct a prognostic index in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy (TAI) using cisplatin suspended in lipiodol.
Methods We analyzed the outcomes in a total of 94 consecutive patients with previously untreated hepatocellular carcinoma who were treated by TAI using cisplatin suspended in lipiodol.
Results Twenty-seven patients (29%) showed complete response and 21 patients (22%) showed partial response, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years and the proportions of survivors at 1, 2, and 5 years were 81.6, 65.2, and 18.3%, respectively. The results of multivariate analysis indicated a significant association of serum albumin $\geq$3.0 g/dL, maximum tumor size $\leq$3.0 cm, absence of ascites, and unilateral distribution of the tumors with a favorable survival. For clinical application, we also propose a prognostic index based on a combination of these prognostic factors. Based on this index, the patients were classified into three groups: those with good, intermediate, and poor prognosis. The median survival times in these three groups were 4.3, 2.7, and 1.1 years, respectively ($p < 0.01$).
Conclusions TAI with cisplatin suspended in lipiodol exhibited favorable tumor efficacy and survival in patients with hepatocellular carcinoma. The prognostic factors identified and the index proposed based on these factors may be useful for predicting life expectancy, determining treatment strategies, and designing future clinical trials.

Keywords Hepatocellular carcinoma · Transcatheter arterial infusion chemotherapy · Cisplatin · Prognosis

Abbreviations

HCC	Hepatocellular carcinoma
TAE	Transcatheter arterial chemoembolization
TAI	Transcatheter arterial infusion chemotherapy
CT	Computed tomography
AFP	Serum alpha-fetoprotein
PIVKA II	Protein induced by vitamin K absence or antagonist-II
CR	Complete response
PR	Partial response

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, and its incidence is continuing to increase worldwide. However, the prognosis of advanced HCC remains unsatisfactory [1]. Curative therapies such as resection, liver transplantation, and local ablative treatments may offer a chance of improved life

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expectancy, but these treatment modalities are applicable to only a small proportion of all HCC patients. Transcatheter arterial chemoembolization (TAE) has been recognized as an effective palliative treatment option for patients with advanced HCC, because two meta-analyses [2, 3] of seven randomized controlled trials [4–10] showed that TAE significantly improves the survival of unresectable HCC patients with preserved hepatic function [1]. Transcatheter arterial infusion chemotherapy (TAI) is also often used for the treatment of advanced HCC, but a consensus regarding the most effective chemotherapeutic regimen has not yet been reached [11, 12]. Lipiodol, a lipid lymphographic agent, is selectively retained by HCC tissues for prolonged periods in comparison with non-cancerous tissues, and is therefore commonly mixed with anticancer agents to allow these agents to be retained for prolonged periods of time in the target tumor [13–15]. In a randomized controlled trial of TAE and TAI with zinostatin stimalamer and lipiodol, TAE did not yield superior survival as compared to TAI in patients with advanced unresectable HCC [16]. Our previous analysis also revealed that TAE did not significantly improve the survival of patients with HCC in comparison with TAI using cisplatin suspended in lipiodol, even though TAE is known to have higher antitumor efficacy than TAI [17]. Thus, TAI may have a higher efficacy on survival compared to TAE. If the appropriate indications for TAI can be expanded, additional embolization may not be necessary in some patients, considering that TAE has more deleterious effects on the liver functions than TAI [17, 18]. However, proper patient selection for TAI with lipiodol has not yet been fully investigated, although those for TAI without lipiodol [19–21] and for TAE [22–24] have been frequently analyzed. Analysis of prognostic factors would suggest appropriate patient selection for TAI. The present study was conducted to investigate the antitumor efficacy of the treatment, and to evaluate a number of variables that may affect survival in patients with HCC treated by TAI using cisplatin suspended in lipiodol; we have proposed a prognostic index in patients treated with TAI based on the results of our analyses.

Materials and methods

Patients

Between October 1987 and May 1996, 94 consecutive patients with previously untreated HCC were treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol at Kumamoto University Hospital, Japan. The study subjects were patients who were judged to be suitable candidates for TAI (Table 1). HCC was diagnosed on the basis of histological examination or distinctive findings on computed tomography (CT) and/or angiography, associated with elevated serum levels of serum alpha-fetoprotein (AFP) or protein induced by vitamin K absence or antagonist-II (PIVKA II). Pretreatment evaluation included a complete medical history and careful physical examination. The laboratory procedures included complete

Table 1 Patient characteristics

	No of patients (%)
Host-related variables	
Age (years)	
Median [range]	64 [41–81]
Gender	
Male	62 (66%)
Blood transfusion	
Present	28 (30%)
Alcohol abuse[a]	
Present	11 (12%)
Smoking habit[b]	
Present	31 (33%)
Hepatitis B surface antigen	
Positive	14 (15%)
Hepatitis C antibody	
Positive	76 (81%)
Ascites	
Present	14 (15%)
Child-Pugh class	
A	45 (48%)
B	48 (51%)
C	1 (1%)
Tumor-related variables	
Number of tumors	
Multiple	53 (56%)
Tumor distribution	
Unilateral	70 (74%)
Maximum tumor size (cm)	
Median [range]	2.9 [1.5–12.0]
Portal vein invasion	
Present	7 (7%)
Alpha-fetoprotein (ng/mL)	
Median [range]	36.9 [1.9–17,100]
PIVKA II (mAU/mL)	
Median [range]	30 [0–6,000]
Other variables	
Modified Japan Integrated Stage	
Median [range]	2 [0–5]

PIVKA II protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake ≥80 g/day for ≥5 years

[b] >20 cigarettes/day for >10 years

differential blood count, biochemistry tests, viral markers, including serum hepatitis B surface antigen and serum hepatitis C antibody, and tumor markers, including the serum levels of AFP and PIVKA II. Before treatment, a chest X-ray and ultrasonography and CT of the abdomen were obtained to evaluate the extent and size of the tumors and to exclude the presence of extrahepatic metastasis. The number, size, and distribution of the tumors were examined by CT and/or angiography. Written informed consent was obtained from all the patients prior to the start of the treatment.

Treatment procedure

Following conventional visceral angiography, TAI was performed by selectively introducing a catheter into the proper, right or left hepatic artery, or a branch of the artery feeding the tumor and injecting cisplatin suspended in lipiodol (iodized oil; Guerbet, Paris, France). The dose of the drug was determined based on the tumor size and liver function. The cisplatin suspension in lipiodol was prepared by the following procedure [25]: cisplatin powder, produced by evaporating water and sodium chloride from cisplatin solution, was sterilized by heating and subsequently suspended in lipiodol with a mortar and pestle under sterile conditions. The content of cisplatin in the lipiodol was adjusted to 20 mg/mL.

After the treatment, follow-up examinations, including CT, tumor marker measurement, and serum biochemistry, were performed, first at one month after the treatment completion and subsequently every 3–4 months. The transcatheter arterial treatments were repeated when relapse of the treated lesions and/or new hepatic lesions were seen.

Evaluation of the antitumor efficacy

The antitumor effect was assessed by contrast-enhanced CT or magnetic resonance imaging at one month after the treatment. Lipiodol accumulation in the tumor was regarded as representing necrotic tissue, because earlier studies have shown that areas on the CT showing lipiodol retention correspond to necrotic areas in the tumors [13–15]. We defined complete response (CR) as disappearance or 100% necrosis of all tumors, and partial response (PR) as >50% reduction and/or necrosis in the sum of all measurable tumors. Progressive disease was defined as more than 25% enlargement in the sum of all lesions and/or the appearance of any new lesions. Stable disease was considered as any disease that did not qualify for classification as CR, PR or progressive disease.

Factors analyzed

The relationships of pretreatment clinical variables to survival were investigated by univariate and multivariate analyses. The pretreatment variables were chosen based on their possible effects on the prognosis and tumor response indicated by previous investigations [1–12, 16–30] or suggested by our own clinical experience. Each of the variables, which were classified as host-related or tumor-related, was divided into two subgroups in accordance with clinically meaningful values for easy application in clinical practice, as shown in Table 2.

Overall survival was measured from the date of initial treatment to the date of death or last follow-up. Survival curves were calculated by the Kaplan–Meier method, and differences in survival were evaluated by the log rank test. The Cox proportional hazard model was used to determine the most significant variables related to survival. Forward and backward stepwise regression procedures based on the partial likelihood ratio were used to determine the major independent predictors of survival. A prognostic index based on the regression coefficients derived from all variables identified by the multivariate analysis was constructed. Stratification of the patients was conducted on the basis of this prognostic index. All p values presented in this report are of the two-tailed type. Differences at $p < 0.05$ were considered to be significant.

Results

Patient characteristics

The characteristics of all the 94 patients are shown in Table 1. There were 62 males (66%) and 32 females (34%), with a median age of 64 (range 41–81) years. There were 45 patients (48%), 48 patients (51%) and 1 patient (1%) with Child-Pugh stage A, B, and C [29], respectively. Fifty-three patients (56%) had multiple tumors, and the median maximum tumor size was 2.9 (range 1.5–12.0) cm. The median modified Japan Integrated Stage [30] was 2 (range 0–5). The median number of courses of TAI was two (range 1–9) during the follow-up period, and the median follow-up duration was 2.5 years (range 0.2–8.4 years). The median dose of cisplatin at first TAI was 50 (range 20–150) mg per treatment.

Treatment efficacy and survival

Twenty-seven patients (29%) showed CR and 21 patients (22%) showed PR, with an overall response rate of 51% (95% confidence interval, 41–61%). The median survival time was 2.5 years, and the proportions of survivors at 1, 2, 3, and 5 years were 81.6, 65.2, 39.8, and 18.3%, respectively (Fig. 1). The cause of death was tumor progression in 47 patients, hepatic failure in 25 patients, rupture of esophageal varices in 4 patients, and other causes in 6

Table 2 Univariate analysis of prognostic factors in patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol

	n	Median survival (years)	2-year survival (%)	Hazard ratio	*p* value
Host-related variables					
Age (years)					
≥60	67	2.5	65		
<60	27	2.6	54	0.98 (0.60–1.59)	0.93
Gender					
Female	32	2.7	66		
Male	62	2.4	60	0.99 (0.61–1.56)	0.97
Blood transfusion					
Present	28	2.5	60		
Absent	66	2.7	63	0.77 (0.48–1.24)	0.28
Alcohol abuse[a]					
Present	11	2.0	55		
Absent	83	2.6	63	0.63 (0.33–1.20)	0.16
Smoking habit[b]					
Absent	63	2.5	59		
Present	31	3.4	69	0.79 (0.50–1.27)	0.33
HBs Ag					
Negative	80	2.5	64		
Positive	14	1.8	46	0.77 (0.40–1.49)	0.45
HCV Ab					
Negative	18	1.9	47		
Positive	76	2.5	65	0.93 (0.53–1.64)	0.81
Ascites					
Present	14	1.4	21		
Absent	80	2.8	69	0.29 (0.16–0.53)	**<0.01**
WBC ($\times 10^4/mm^3$)					
≤4.0	51	2.5	61		
>4.0	43	2.5	64	0.76 (0.49–1.19)	0.23
Hemoglobin (g/dL)					
<10	17	2.4	59		
≥10	77	2.6	63	0.69 (0.40–1.19)	0.18
Platelet ($\times 10^4/mm^3$)					
<7.5	36	2.5	67		
≥7.5	58	2.5	59	0.89 (0.57–1.37)	0.59
Total bilirubin (mg/dL)					
≥2.0	13	1.8	46		
<2.0	81	2.7	65	0.59 (0.32–1.09)	0.09
Albumin (g/dL)					
<3.0	33	1.6	35		
≥3.0	61	4.0	76	0.29 (0.18–0.47)	**<0.01**
AST (U/L)					
≥85	24	2.4	58		
<85	70	2.8	63	0.63 (0.38–1.04)	0.07
ALT (U/L)					
≥92	21	2.4	57		
<92	73	2.7	63	0.74 (0.44–1.24)	0.25
LDH (U/L)					
≥500	9	1.8	44		
<500	85	2.5	64	0.76 (0.36–1.58)	0.46
Prothrombin time (%)					
<70	41	2.4	58		
≥70	53	2.7	65	0.93 (0.60–1.45)	0.76
ICG R15 (%)					
≥30	46	2.2	52		
<30	43	3.4	71	0.68 (0.43–1.07)	0.09
Tumor-related variables					
Number of tumors					
Multiple	53	2.0	51		
Single	41	2.8	76	0.63 (0.41–0.98)	**<0.05**
Tumor distribution					
Bilateral	24	1.1	27		
Unilateral	70	2.8	73	0.39 (0.24–0.65)	**<0.01**
Maximum tumor size (cm)					
>3.0	40	1.6	42		
≤3.0	54	3.2	76	0.41 (0.26–0.66)	**<0.01**
Portal vein invasion					
Present	7	1.0	17		
Absent	87	2.6	65	0.36 (0.15–0.84)	**<0.05**
Alpha-fetoprotein (ng/mL)					
≥100	46	2.4	57		
<100	48	2.6	67	0.66 (0.42–1.02)	0.06
PIVKA II (mAU/mL)					
≥100	14	1.1	34		
<100	80	2.7	67	0.53 (0.29–0.97)	**<0.05**

p values lesser than 0.05 are given in bold

HBs Ag hepatitis B surface antigen, *HCV Ab* hepatitis C antibody, *WBC* white blood cell count, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactic dehydrogenase, *ICG* indocyanine green test, *PIVKA II* protein induced by vitamin K absence or antagonist-II

[a] Ethanol intake ≥80 g/day for ≥5 years

[b] >20 cigarettes/day for >10 years

patients. Neither severe toxicity including renal dysfunction or thrombocytopenia, nor complication or treatment related death were seen in the present study.

Univariate and multivariate analysis

The median survival times, two-year survival, hazard ratios and *p* values of the survival time for univariate analysis are shown in Table 2. Among the host-related factors, absence of ascites and a serum albumin level of >3.0 g/dL were

significantly associated with a longer survival time. Among the tumor-related factors, single nodule, unilateral distribution of tumors, maximum tumor size <3.0 cm, absence of portal vein invasion, and PIVKA II level <100 mAU/mL were significantly associated with a longer survival time. The results of multivariate analysis using the Cox proportional hazard model are shown in Table 3. In the multivariate analyses, only those variables identified as significant by the univariate analysis were entered. Serum albumin ≥3.0 g/dL, maximum tumor size <3.0 cm, absence of ascites, and unilateral distribution of the tumors were significantly associated with favorable survival.

Risk groups based on the regression model

For the clinical application of these findings, a prognostic index was calculated based on the regression coefficients derived from the four variables identified by multivariate analysis (Table 3), as follows: prognostic index = score for albumin (0 for ≥3.0, 1 for <3.0 g/dL) + score for ascites (0 for absence, 1 for presence) + score for maximum tumor size (0 for ≤3.0, 1 for >3.0 cm) + score for tumor distribution (0 for unilateral, 1 for bilateral). The index values ranged from 0 to 4. The patients were then classified into three groups according to the prognostic index, as follows: good prognosis group (Group A: prognostic index = 0, $n = 31$ patients) (equivalent to patients with none of the four prognostic factors); intermediate prognosis group (Group B: prognostic index = 1, $n = 28$ patients) (equivalent to patients with one of the four prognostic factors); poor prognosis group (Group C: prognostic index ≥ 2, $n = 35$ patients) (equivalent to patients with two or more of the four prognostic factors). The survival curves for the three groups are shown in Fig. 2. The median survival times in the good, intermediate, and poor prognosis groups were 4.3, 2.7, and 1.1 years, respectively. There were significant differences in the survival time among the three groups ($p < 0.01$).

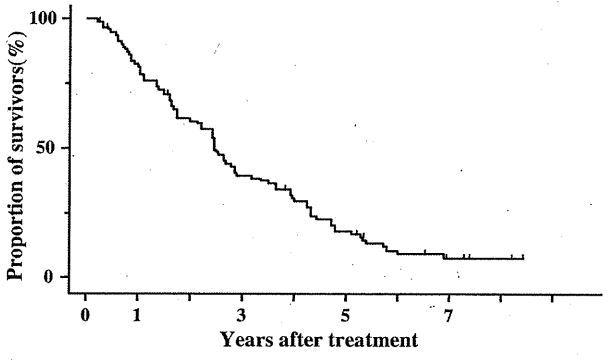

Fig. 1 Overall survival curve for all patients with hepatocellular carcinoma treated by transcatheter arterial infusion chemotherapy using cisplatin suspended in lipiodol. *Tick marks* indicate censored cases

Discussion

TAE has been widely used for cases with unresectable HCC and is currently the mainstay of non-surgical treatment for HCC, because it has been shown to exert a marked antitumor effect against HCC and can be administered for any type of HCC, regardless of the size, location or number of tumors [1]. In addition, the survival benefit of this treatment modality has been verified by two meta-analyses [2, 3] of seven randomized controlled trials [4–10]. However, TAE has deleterious effects on liver functions, thereby impairing the baseline prognosis. On the other hand, TAI has milder hepatotoxicity, but also shows a lower antitumor efficacy against advanced HCC than TAE. However, in a randomized controlled trial of TAE versus TAI with zinostatin-stimalamer and lipiodol, TAI and TAE were reported to yield comparable survival [16]. Moreover, the result of our retrospective analysis of TAE versus TAI using cisplatin–lipiodol suspension indicated similar outcomes for the two modalities [17]. From the results of these two studies, we could not conclude that additional embolization is not necessary for the treatment of advanced HCC, but there may be a subset of patients of advanced HCC in which TAI alone may yield sufficient treatment efficacy and survival. Therefore, this analysis of prognostic factors was carried out to enable identification of appropriate candidates for TAI using cisplatin–lipiodol suspension among HCC patients with no prior treatment. This single-institution study was undertaken using a unified method for tumor staging and identical procedures for treatment, follow-up, and supportive care throughout the duration of the study, to enable us to obtain reliable results for confirming important

Table 3 Significant prognostic factors determined by multivariate analysis with the Cox proportional hazard model

Variable	Coefficient	Hazard ratio (95% confidence intervals)	p value
Albumin ≥3.0 g/dL	0.94	0.39 (0.23–0.66)	<0.001
Maximum tumor size ≤3.0 cm	1.01	0.37 (0.19–0.69)	0.001
Absence of ascites	0.81	0.45 (0.11–0.40)	0.002
Unilateral tumor distribution	0.77	0.46 (0.27–0.79)	0.004

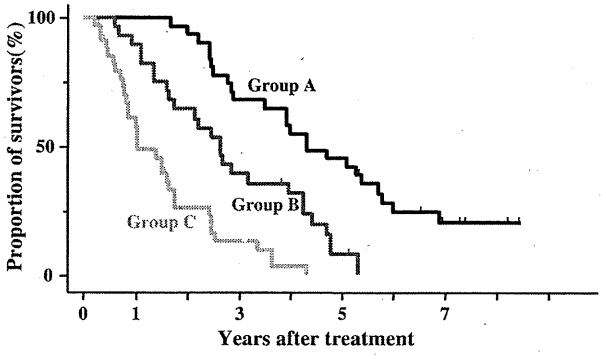

Fig. 2 Survival curves for the three groups determined by a prognostic index. *Group A* good prognosis (31 patients), *Group B* intermediate prognosis (28 patients), *Group C* poor prognosis (35 patients). *Tick marks* indicate censored cases

prognostic factors, predicting life expectancy and designing future clinical trials of TAI for HCC.

In this study, cisplatin was administered as the anticancer agent for TAI. Cisplatin has been reported to exert its actions by binding to the DNA in cancer cells, inhibiting DNA synthesis and subsequent cellular division. It is one of the key drugs for advanced HCC, that constituted a component of the combined chemotherapeutic regimen used in three of the seven randomized controlled trials of TAE reported until date [6, 7, 9]. In Japan, a favorable tumor response (33.8%) was reported in a clinical study of intra-arterial administration of cisplatin for advanced HCC [21], and the treatment has been approved for the treatment of HCC by the Ministry of Health, Labour and Welfare of Japan. Lipiodol has been used as a carrier for anticancer agents in targeting chemotherapy [13–15], and a suspension of cisplatin powder in lipiodol was used in this study. It has been reported that stronger antitumor effect is obtained by hepatic arterial administration of a combination of lipiodol and an anticancer agent than by that of an anticancer agent alone [26]. Recently, a lipophilic cisplatin derivative that can be suspended in lipiodol, SM-11355, was reported to show promising tumor efficacy (CR rate: 56%) in a phase II trial, and further trial is ongoing [27]. Therefore, combined therapy with cisplatin and lipiodol has been expected to become established as a valid option for the treatment of HCC. The response rate (51%: 95% confidence interval, 41–61%) at one month obtained in this study was more favorable than that in a clinical study of cisplatin alone, because TAI with an emulsion of an anticancer agent and lipiodol could be expected to exert more potent effects than an anticancer agent alone. However, follow-up at one month might be insufficient for evaluation of the rate/pattern of recurrence of HCC.

The median survival time and survival rates at two years in the current study were 2.5 years and 65.2%, respectively. These results were comparable or superior to those of TAE reported from the aforementioned seven randomized controlled trials [4–10]. Although the study was based on a retrospective cohort design, the treatment efficacy of TAI with cisplatin–lipiodol suspension was promising and comparable to that of TAE for HCC.

In regard to the host-related factors, absence of ascites and a serum albumin level >3.0 g/dL were found to be favorable prognostic factors by multivariate analysis. Ascites and albumin are the most important factors to consider when evaluating the hepatic reserve, being included in both the Okuda staging system [28] and Child-Pugh classification [29], and have been shown to be prognostic factor in previous studies of patients with advanced HCC [19, 20, 22–24]. In regard to the tumor-related factors, a maximum tumor size $\leq$3.0 cm and unilateral distribution of the tumors were identified as being significantly associated with a longer survival time by multivariate analysis. Increased tumor size and bilateral distribution of tumors are the well-known unfavorable prognostic factors in HCC patients, and have been shown to be correlated with increased tumor volume and poorer differentiation of HCC, which reflect a more advanced stage and higher malignant potential of the tumors [22]. However, these prognostic factors for TAI with lipiodol in this study were similar to those identified for TAI without lipiodol [19–21] or TAE in previous reports [22–24], and no specific prognostic factors for TAI could be identified in this study.

For clinical application of these findings, we propose a prognostic index based on the independent prognostic factors identified in this study. Patients could be classified into three groups: those with good, intermediate, and poor prognosis ($p < 0.0001$) (Fig. 2). This index consists of both hepatic reserve and tumor stage, like the modified JIS score [30], and it differs from the Child-Pugh stage or TNM stage which are, respectively, based on either only the hepatic reserve or tumor stage. An index based on both the hepatic reserve and tumor stage might enable a more accurate prediction of life expectancy and stratification of the group into more distinct prognoses. This index can be easily calculated, because it is based on variables obtained during routine examinations before TAI. It can, therefore, be used to stratify patients with HCC before TAI according to the predicted survival. Accordingly, patients with good prognosis may obtain sufficient treatment efficacy and survival with TAI alone. In contrast, patients with a poor prognosis may be treated with supportive care only because of the extremely short median survival (1.1 years) expected, or may be treated other more aggressive treatments, such as more intensive chemotherapy. Recently, systemic chemotherapy for advanced HCC has become an important treatment modality, because sorafenib has been proven to confer a survival benefit and to show promise as a standard

treatment for patients with advanced HCC [31]. To improve the treatment efficacy, further chemotherapy regimens, such as the combination therapy comprising TAI with cisplatin suspended in lipiodol and sorafenib or other molecularly targeted agents, remain as challenges to be met following further detailed investigations. These findings may be helpful in predicting the life expectancy in HCC patients treated with TAI and provide more information to stratify patients in future TAI trials. It is also important to validate this prognostic index by applying it to other populations of HCC patients.

In conclusion, TAI with cisplatin suspended in lipiodol exhibited favorable tumor efficacy and survival in patients with HCC. Although no specific prognostic factors for TAI could be identified in this study, the results of the prognostic factors and the prognostic index may be helpful for predicting life expectancy, determining the most appropriate treatment strategies, and designing future clinical trials.

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Chronic hepatitis C viral infection reduces NK cell frequency and suppresses cytokine secretion: Reversion by anti-viral treatment

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ABSTRACT

Impaired activity of NK (natural killer) cells has been proposed as a mechanism contributing to viral persistence and chronic infection in hepatitis C (HCV) infection. We aimed to assess the impact of HCV infection on NK cells regarding frequency, subset distribution, and cytotoxic and cytokine secretion functions, as well as IFN-α and ribavirin therapeutic effects on NK cells. Significant reduction of total NK frequency and the $CD56^{dim}16^{+}$ subset was observed in chronic HCV patients. IFN-γ expression upon stimulation with K562 was severely suppressed but cytotoxicity measured by CD107a expression was maintained. These adverse effects were reversed after treatment with pegylated IFN-α and ribavirin; however, these skewed functions were not recovered in treatment-resistant patients. Thus, HCV chronic infection severely affects NK functions, except for cytotoxicity. Altered NK cell frequency and cytokine secretion by HCV infection may contribute to impaired cellular immune response and virus persistence.

Introduction

Hepatitis C virus (HCV) infects about 3% of the global population, and it is estimated that only 20% of newly infected individuals will develop sufficient immune response to clear the infection [1]. Chronic infection with HCV results in degenerative liver disease that might end in cirrhosis or hepatocellular carcinoma [2]. Natural killer (NK) cells are considered to be the first line of defense against viral infections and cancer through their rapid cytotoxic activity and cytokine production [3]. Several controversial studies have reported the interaction between NK cells and HCV. These effects include suppression of HCV viral replication by NK cells, downregulation of NK natural cytotoxicity receptors by HCV, and cross-linking of HCV-E2 protein to the tetraspanin CD81 [4–7]. Significant numbers of NK cells are known to reside in the liver and to interact with virus infected hepatocytes [8,9]. A recent report showed that NK cells are polarized towards cytotoxicity during HCV infection, which could play a direct role in liver injury and the persistence of HCV infection [10]. These studies emphasize the important role played by NK cells against HCV infection, as well as the immune evasion mechanisms implemented by HCV.

Combination therapy of pegylated IFN-α and ribavirin is now becoming the standard therapy against chronic HCV infection; however, the number of responding patients is limited to 50–70% [11,12], and the detailed molecular mechanisms of action of IFN-α are insufficiently understood. IFN-α anti-viral activity is not directly related to the virus or replication complex, but rather acts by inducing IFN-stimulated genes, which establish a non-virus-specific anti-viral state within the cell [13,14]. Enhancement of memory T-cell proliferation, prevention of T-cell apoptosis, stimulation of NK cell activation and dendritic cell maturation by Type 1 IFNs have been reported [15]. IFN-α has also complex and indirect effects on the immune system, which modify NK cell numbers and functions [7]. As NK cells are known to have strong anti-HCV activity [6,16], recovered NK cells have a key role in suppressing HCV replication after IFN-α treatment.

As most of the studies either assess treatment effect on NK cells in vitro, or compare different groups of patients, we aimed to assess the impact of HCV infection on NK cell activities through analyzing the same patients before and after treatment, excluding individual variations. We found that combination therapy of pegylated IFN-α and ribavirin reversed NK subtype distribution and functions in HCV-eliminated patients. Interestingly, relapsing patients failed to recover NK functions. These results suggest the

Abbreviations: HCV, hepatitis C; NCR, natural cytotoxicity receptor; PFN, perforin; LAMP1, lysosomal-associated membrane protein 1; Pre-Tx, before starting treatment; Tx-End, on finishing treatment; Post-Tx, 24 weeks after finishing treatment.

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contribution of NK alteration caused by HCV infection to the impairment of cellular immune response, which might result in chronic disease.

Materials and methods

Patients and samples. Peripheral blood (PB) samples were collected from 29 chronic HCV-infected patients attending the Department of Gastroenterology and Hepatology, Kumamoto University Hospital, Kumamoto, Japan. No patients were receiving anti-viral treatment when enrolled in the study. All 29 patients were biopsied before starting the treatment course. Out of 29 patients, 12 were followed up after starting pegylated IFN-α and ribavirin treatment. These 12 patients were followed up by sample collection and analysis at the end of treatment, and 6 months after finishing the treatment. Twenty-two age-matched healthy subjects were attending Kumamoto City Medical Association Health Care Center (Supplementary Table 1). All samples were collected with written informed consent, and the study was approved by the Ethics Committee of Kumamoto University Graduate School of Medical Science (Approval No. 657, issued 10 May, 2007).

Viral genotyping, viral load measurements, and treatment course. HCV RNA was quantified using The COBAS TaqMan HCV Test v2.0 (Roche Diagnostics, Indianapolis, IN). The detection level was 0.2 KIU/ml. The test was carried out before starting treatment, at the end of treatment, and 24 weeks after finishing treatment. Genotyping was performed using the HCV genotype primer kit from Bio Medical Laboratories (BML, Japan). Patients received Peg-IFN-α2a (Chugai Pharmaceutical, Japan) at 180 μg/week; Peg-IFN-α2b (Schering-Plough, Japan) at a weekly dose ranging 60–150 μg, and ribavirin (Roche, Schering-Plough) at a daily dose ranging 600–1000 mg/day, depending on body weight, for a period ranging 24–72 weeks (depending on HCV genotype or viral load).

Cell separation and culture stimulation. Peripheral blood mononuclear cells (PBMCs) were separated by lymphocyte separation medium (LSM Cappel, Aurora, OH) from heparinized fresh peripheral blood samples. The K562 cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan), and cultured in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS. The effector:target cell ratio was adjusted to 10:1. Cells were suspended in RPMI and incubated in a 5% CO_2 incubator for 1 h, after which BrefeldinA (10 μg/ml) was added to detect intracellular IFN-γ, or Monensin (6 μg/ml, Sigma) was added to prevent the degradation of re-internalized CD107a (degranulation marker) proteins from the surface [17]. The cells were then incubated for a further 5 h.

Flow cytometry. The following mAbs were used in the study: CD3-PECy7 (UCHT1) and anti-Perforin (PFN)-FITC (dG9) from e-Bioscience (San Diego, CA); CD56-APC (NKH-1), NKG2D-PE (ON72), NKG2A-PE (Z199), NKp30-PE (Z25), and NKp46-PE (BAB281) from Beckman Coulter (Paris, France); CD16-Pacific Blue (3G8) from Biolegend (San Diego, CA); CD94-FITC (HP3D9), CD107a-PE (H4A3), and anti-IFN-γ FITC (4S.B3) from BD Pharmingen (San Diego, CA). For surface staining, cells were stained and incubated for 30 min on ice in the dark. For intracellular staining, cells were then washed in FACS washing medium (PBS, 0.1% NaN_3, and 3% FCS) fixed with 4% paraformaldehyde washed and permeabilized using 0.1% Saponin (Sigma), and then stained with mAbs. Cells were re-suspended in FACS washing medium and analyzed by LSRII (BD Bioscience, San Jose, CA) and FlowJo software (Tree Star, San Carlos, CA).

Statistical analysis. Graphpad Prism 5 (Graphpad Software, Inc.) was used to calculate non-parametric Mann–Whitney test to compare Patients before treatment to the control group. Paired *t*-test was used to calculate the modifications that occurred between stages in the same patient.

Results

HCV infection reduces NK cell frequency and induces shifting among its subsets

We first analyzed total NK cell number and subset frequency in PBMC of HCV-infected patients without treatment (Fig. 1A). Both the percentage and absolute number (Fig. 1B and C) of NK cells were significantly decreased in HCV patients compared to normal controls (percentage: $P = 0.0005$. Absolute number: $P = 0.0002$). Within the NK pool, the $CD56^{bright}$ subset percentage was significantly higher among HCV-infected patients ($P = 0.007$), but not the absolute number. The $CD56^{dim}16^{+}$ subset showed significant decrease among HCV patients both in percentage ($P = 0.0001$), and absolute number ($P < 0.0001$). As for the $CD56^{dim}16^{-}$ subset, also known as the activated NK subset [18,19], the percentage significantly increased in HCV patients ($P < 0.0001$) as well as the absolute number ($P = 0.03$). The $CD56^{-}16^{+}$ subset showed a significant rise among HCV-infected patients in percentage ($P = 0.02$), but not in absolute number. There were no significant correlations with viral load, genotype, AST/ALT levels, and degree of activity or liver fibrosis (data not shown). These results show that HCV affects mainly the $CD56^{dim}16^{+}$ subset, causing its decrease, leading to disruption in other subset percentages.

NCRs' and C-lectin-type receptor expression by NK cells in HCV infection

Next, we analyzed the expressions of NCRs (NKp30 and NKp46), inhibitory receptors (CD94 and NKG2A), and activation receptor NKG2D. Overall, total NK cells showed no significant difference in any of these receptors percentages (Supplementary Fig. 1A), except for NKG2D, which showed significant downregulation during HCV infection ($P < 0.0001$) (Fig. 2A and B). Detailed analysis of NK subsets showed downregulation of all NCRs by $CD56^{dim}16^{-}$ subset (Supplementary Fig. 1B).

HCV infection suppresses PFN and IFN-γ by NK cells, but degranulation ability is maintained

Cytotoxic function of NK cells depends on intracellular functional molecules, so we analyzed Perforin expression in NK cells. As shown in Fig. 2A, HCV infection significantly downregulated PFN expression by total NK cells ($P < 0.0001$). NK subset analysis (Fig. 2B) revealed that this downregulation is confined only to the $CD56^{dim}16^{+}$ subset ($P = 0.0006$). CD107a is a degranulation marker that is equivalent to cytotoxic function [17]. NK cell degranulation was significantly higher among HCV patients (Fig. 2C, $P = 0.002$). Next, we analyzed IFN-γ expression by NK cells stimulated with K562 cells. HCV infection markedly suppressed IFN-γ upregulation (Fig. 2C) ($P = 0.001$). The positive CD107a and IFN-γ subset was found to be $CD56^{dim}16^{-}$ (data not shown). The correlation between CD107a and IFN-γ expressions by the $CD56^{dim}16^{-}$ subset in normal controls was statistically significant ($r = 0.6$, $P = 0.002$), while this correlation was lost in HCV-infected subjects ($r = 0.1$, $P = 0.4$).

Pegylated IFN-α treatment modification of NK cells

We followed up 12 patients until 24 weeks after finishing the treatment course. Samples were collected from these 12 patients before starting treatment (Pre-Tx), at the end of treatment (Tx-End), when 12 subjects were negative for HCV RNA, and 24 weeks after finishing treatment (Post-Tx), when three subjects relapsed to HCV RNA positive. NK percentage did not change

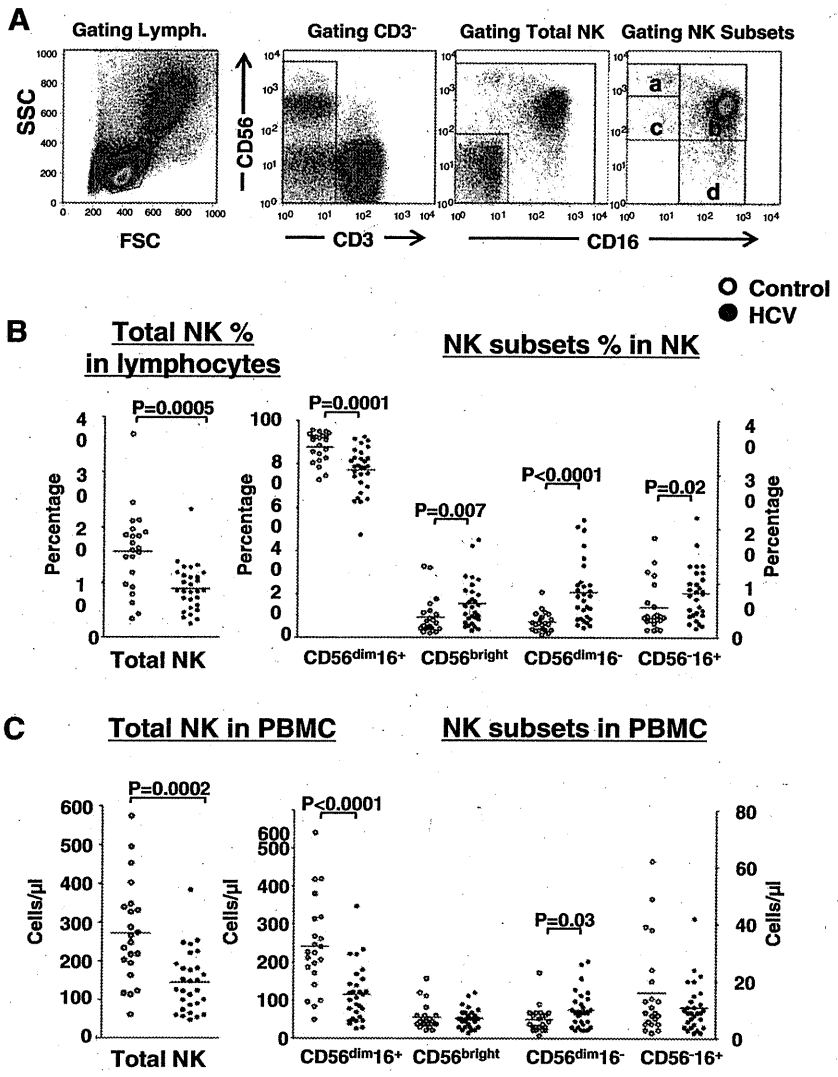

Fig. 1. Frequency and absolute number of NK cells in chronic HCV patients. (A) Lymphocytes were gated through SSC and FSC, followed by gating of $CD3^-$ events, and gating of $CD56^+$ and/or $CD16^+$ events. NK subsets were then gated on CD56 versus CD16. The plot shows the gating of (a) $CD56^{bright}$ subset, (b) $CD56^{dim}16^+$ subset, (c) $CD56^{dim}16^-$, and (d) $CD56^-16^+$ subset. (B) Total NK cell (left) percentage in lymphocytes and the 4 mentioned subset percentages (right) in NK compartment. (C) Absolute NK cell count. In panels (B) and (C), $CD56^{dim}16^+$ is plotted against the left *Y* axis, while the other subsets are plotted against the right *Y* axis. Plots show the results in healthy controls ($n = 22$) (open circles) and chronic HCV-infected subjects ($n = 29$) (closed circles). All figures show the means ± SD.

significantly throughout the follow-up period; however, the absolute numbers of NK cells (Fig. 3A) increased significantly ($P = 0.04$). The $CD56^{bright}$ (Fig. 3B) percentage increased significantly from Pre-Tx to Tx-End and decreased significantly again from Tx-End to Post-Tx ($P = 0.001$ and $P = 0.0002$, respectively). $CD56^{dim}16^+$ (Fig. 3C) showed a significant increase from Tx-End to Post-Tx both in percentage and absolute number ($P = 0.0003$, 0.02, respectively). $CD56^{dim}16^-$ (Fig. 3D) percentage decreased significantly from Tx-End to Post-Tx ($P = 0.02$), but not in absolute value. The $CD56^-16^+$ subset showed a significant decrease from patients to Tx-End in percentage, but not in absolute value ($P = 0.04$). These results show that treatment directly affects the NK cell compartment through expansion of the $CD56^{dim}16^+$ subset, resulting in the return of other NK subsets to normal by 6 months after treatment.

Anti-viral treatment recovered NK function

NKG2D (Fig. 4A) expression showed a significant increase in percentage only from Pre-Tx to Post-Tx ($P = 0.001$). PFN expression was raised significantly from Pre-Tx to Post-Tx ($P = 0.002$) (Fig. 4B), showing that anti-viral treatment upregulates PFN expression by NK cells. Although the percentage of CD107a expression decreased upon treatment, since all NK cell are increased, CD107a-expressing cells did not show any significant change in absolute cell count throughout the treatment course, or 24 weeks later (data not shown). The IFN-γ-expressing NK cell percentage rose significantly from Pre-Tx to Post-Tx ($P = 0.01$) (Fig. 4D).

Absence of NK treatment modifications in relapsed patients

Three cases relapsed to positive HCV RNA 24 weeks after treatment. Although the case number does not enable statistical analysis, the pattern of results seen in relapsed cases suggests a specific tendency. The results showed NK frequency reduction to Pre-Tx levels as well as subset distribution and PFN expression (Supplementary Fig. 2A, B, and C).

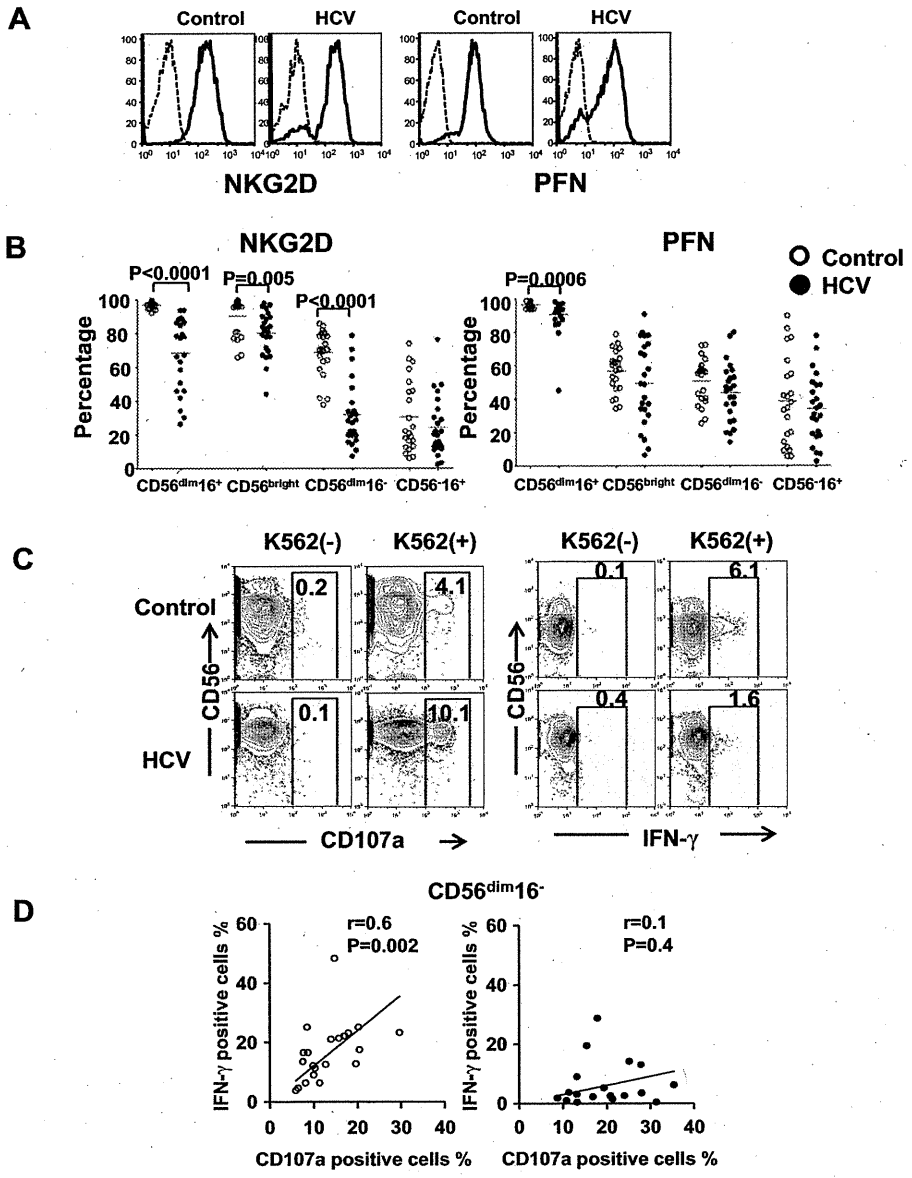

Fig. 2. Expression of NKG2D, PFN, CD107a degranulation marker and IFN-γ by NK cells in chronic HCV patients. (A) Histogram showing the expression of NKG2D and PFN in healthy control and HCV-infected patients NK cells. Isotype control is shown in dotted line. (B) Vertical scatter showing the percentage of NKG2D and PFN positive NK cells in different NK subsets in controls and HCV patients. Control: open circles, HCV patients: closed circles. (C) Contour plots showing Gated NK cells expression of CD107a and IFN-γ with and without stimulation using K562 cells, in control and HCV patients. Plots are displaying the positive cells percentage. (D) Scatter plots showing the correlation between CD107a and IFN-γ expression by $CD56^{dim}16^{-}$ subset in normal controls (left) and HCV patients (right).

Discussion

In this study, we investigated the alteration by chronic HCV of NK cell frequency, subset reconstitution, NK receptor expression, and NK functions, as well as the modification by anti-viral treatment. Study subjects consisted of both genotype 1 and 2. However, our data showed no significant difference between the two genotypes regarding NK frequency, receptors expression, or functions (data not shown). Our data confirm a significant reduction in NK cell frequency and quantitative imbalance of NK cell subsets in HCV-infected patients, which was reversed by anti-viral treatment. This reduction was localized to the $CD56^{dim}16^{+}$ subset. Detailed analysis revealed that the expression of NK activation receptor (NKG2D), PFN, and IFN-γ production were suppressed in chronic HCV patients. It is of interest that the expression of a degranulation marker, CD107a, was not influenced by chronic HCV infection. Follow-up study showed that the expression of these receptors was recovered in treated patients who eliminated HCV. Our results suggest that chronic HCV infection affects the recognition of target cells by NCRs and IFN-γ production of NK cells, and contributes to chronic HCV infection.

We analyzed in detail how the reduction in NK cell numbers and the shift of NK subsets may lead to functional deficits within the total NK cell population of chronic HCV patients. Our results show that HCV infection downregulated NKG2D by NK cells (Fig. 2). As both cytotoxicity and IFN-γ production are regulated by NKG2D-DAP10 signaling, it is reasonable that the expressions of CD107a and IFN-γ are positively correlated in normal

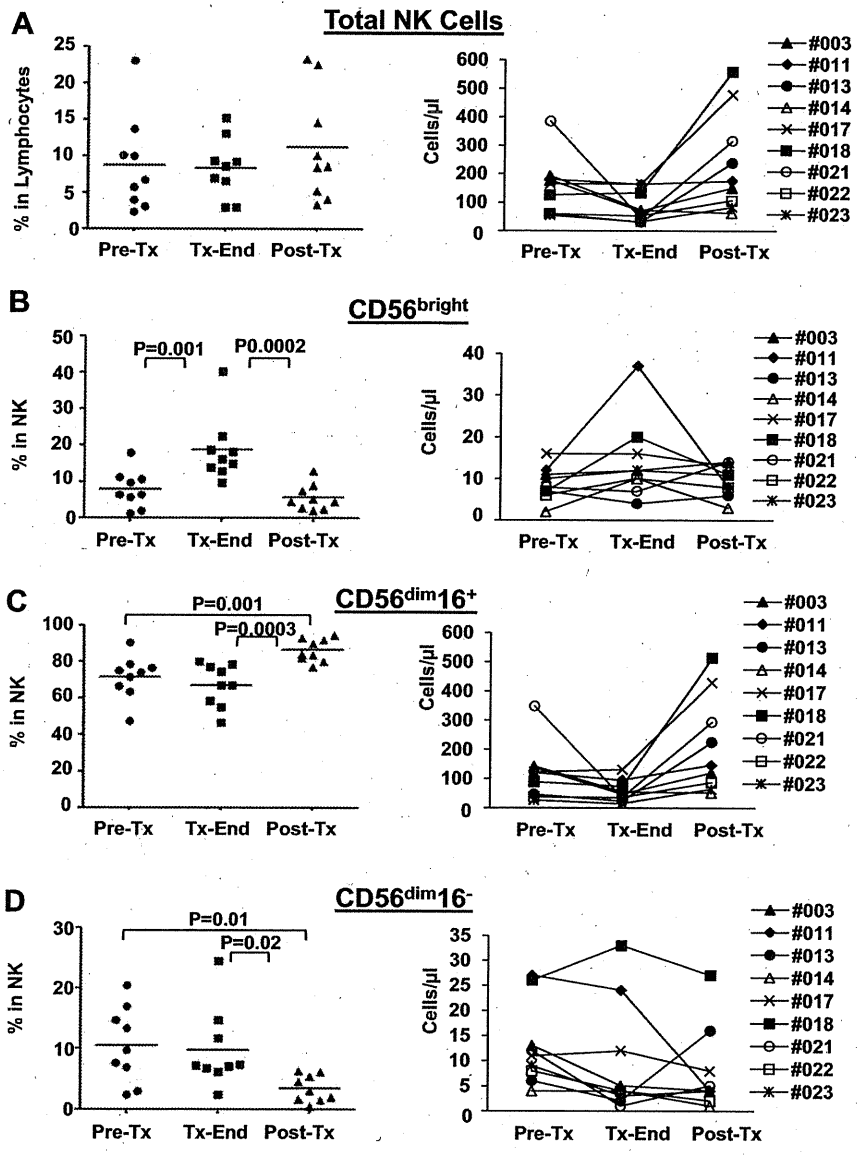

Fig. 3. Recovery of NK cell numbers and component by anti-viral treatment. Anti-viral treatment reverses the reduction in NK cell count. Vertical scatter plot showing the modification in percentage and cell count before (Pre-Tx) (closed circles), at the end (Tx-End) (closed squares), and 6 months (Post-Tx) (closed triangles) after finishing treatment in (A) total NK, (B) $CD56^{bright}$ subset, (C) $CD56^{dim}16^{+}$ subset, and (D) $CD56^{dim}16^{-}$ subset ($n = 9$).

individuals; however, it is of interest that CD107a expression upon stimulation remained high (data not shown) although NKG2D and IFN-γ expressions were suppressed in HCV patients. Since NK cell activity is regulated by the fine balance of the activating signal via NKG2D and inhibitory signals via CD94/NKG2A, modulation of the interplay between inhibitory and activation signals by HCV can markedly change the properties of NK cell function [9,20].

We followed the NK cell status of the patients before and after pegylated IFN-α and ribavirin treatment, and assessed the treatment results and status of NK cells 24 weeks after treatment. Nine of 12 patients succeeded in eliminating HCV. Overall NK cell levels, the subpopulation of NK cells, expression of NK cell receptors, and function of NK cells (CD107a and IFN-γ expressions after co-culture with K562) were recovered to normal levels. In contrast, patients relapsing after 24 weeks had not recovered these parameters. These results indicate that HCV activity affects NK cell numbers and function; however, HCV virions did not affect NK cell activation or effector function either directly or indirectly in vitro [21], indicating that HCV virions do not modulate NK function in the acute phase of infection. As regards chronic HCV infection, there are multiple reports that NK cell function is altered by HCV infection [22,23]. HCV-E2 glycoprotein is known to bind to the tetraspanin CD81 expressed by NK cells and inhibits NK cell activation and IFN-γ production [5]. In addition, HCV NS3/4A protease has been shown to interfere with IFN induction by preventing the RIG-1 helicase pathway [24] as well as IRF3 phosphorylation [25], which favors viral propagation and presumably HCV chronic infection. Elucidation of these mechanisms may lead to NK cell-mediated therapy against HCV.

In this study, we were able to exclude the individual variation in NK function and frequency, which caused the controversy in previous reports. We expected that the NK subset and function may differ between treatment responders and relapsing patients at the end of treatment, from which we can predict therapeutic performance; however, we found no relation between them at the end of treatment. This may be because the influence of administrated

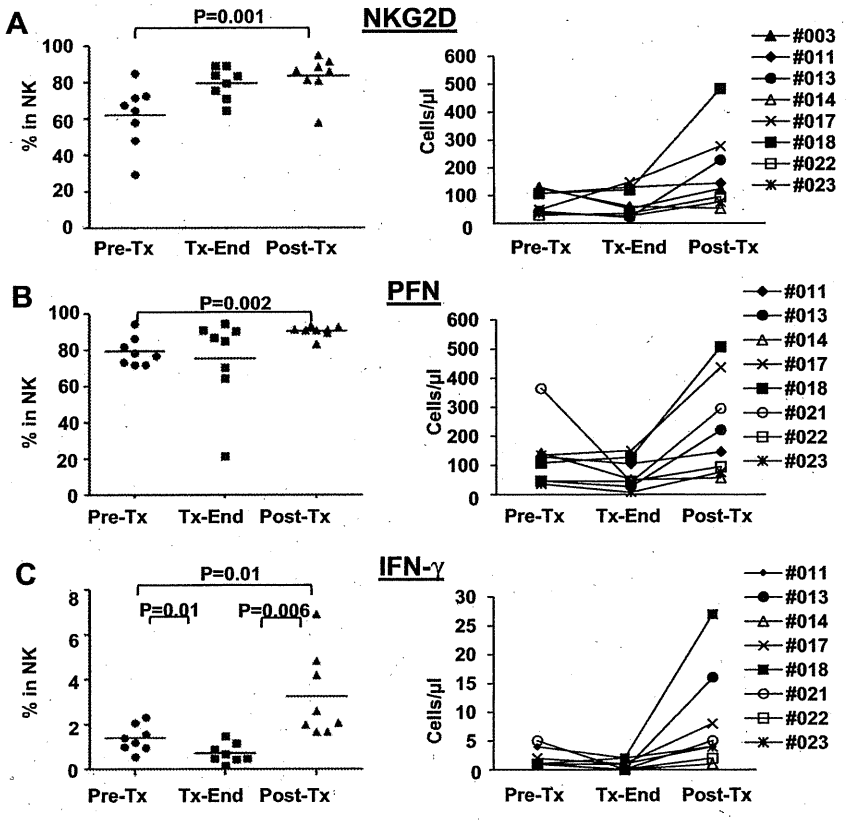

Fig. 4. Recovery of NK cell functional markers by anti-viral treatment. Vertical scatter plots for (A) NKG2D ($n = 8$), (B) PFN ($n = 8$), and (C) IFN-γ ($n = 8$) expressing NK cell percentage and cell count in patients before (Pre-Tx) (closed circles), at the end (Tx-End) (closed squares), and 6 months (Post-Tx) (closed triangles) after finishing treatment (left).

IFN-α was persisting. It is possible that recovered NK cells suppress the recurrence of HCV, since NK cells have anti-HCV activity [6]. Further detailed follow-up studies are expected to show phenotypical and functional differences between responders and non-responders, and if non-responders or early relapse can be predicted by changes in the NK cell fraction in advance, it will be a strong benefit for HCV patients.

Several studies suggest that HCV viral persistence may be associated with decreased NK cell numbers and defective NK cell responses. The reason for the decreased frequency of NK cells in chronic HCV patients is currently unknown, although possible explanations may include death or turnover, or decreased production of NK cells [23]. In addition, as cross-linking of HCV-E2 glycoprotein to CD81 inhibits the activation and proliferation of NK cells [5], it remains possible that high local concentration of envelope protein of HCV may alter NK cell function and frequency. NK cell development, maturation and function are strongly influenced by immunoregulatory cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21, among which, Meier et al. showed that serum levels of IL-15 were significantly reduced in HCV patients and that IL-15 rescued NK cells of HCV patients from apoptosis and promoted proliferation and NK cell function ex vivo [26]. In addition, IFN-αβ is known to upregulate IL-15 production in HCV patients, and the serum level of IL-15 is significantly higher in early virological responders, whereas late virological responders show a low IL-15 level. Thus, reduced serum levels of IL-15 may also contribute to a common mechanism underlying NK cell abnormalities in HCV patients and IL-15 treatment may be beneficial for the eradication of HCV.

Conclusions

In conclusion, our study revealed that HCV chronically infected patients show conserved cytotoxic activity and dysfunctional cytokine production, which are likely due to the direct effects of HCV and may be key factors contributing to the virus persistence of chronic hepatitis. Cytokine treatment, such as IL-12, IL-15 and IL-21, may recover IFN-γ production by NK cells and support the effects of pegylated IFN-α and ribavirin treatment. Such study will be useful for the development of selective and effective therapies for chronic hepatitis C infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.008.

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