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

Results

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

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

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

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

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

Table 2 Characteristics of HCC patients before HCC treatment

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

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

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

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

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

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

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

SFC Spot-forming cells

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

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

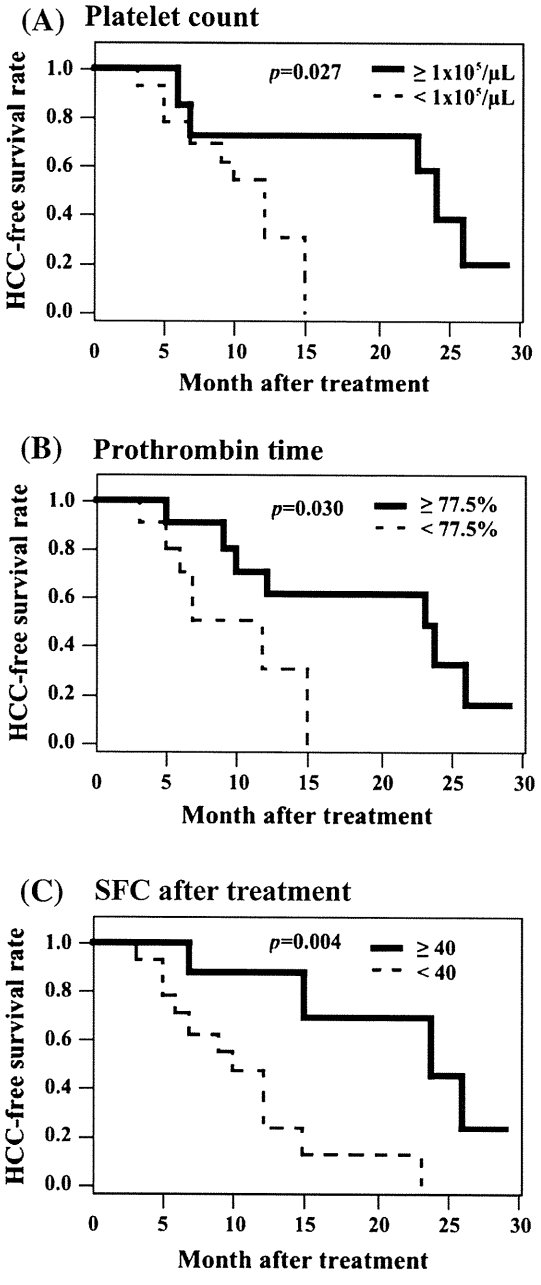

Fig. 1 Kaplan–Meier curves of HCC-free survival rate. In univariate analysis, platelet count, prothrombin time, and the tumor-associated antigen-specific $CD8^+$ T-cell response were found to be prognostic factors for the HCC-free period after treatment. Kaplan–Meier curves representing the relationship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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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Infection of B Cells With Hepatitis C Virus for the Development of Lymphoproliferative Disorders in Patients With Chronic Hepatitis C

Momoko Inokuchi,[1] Takayoshi Ito,[1]* Manabu Uchikoshi,[1] Yuu Shimozuma,[1] Kenichi Morikawa,[1] Hisako Nozawa,[1] Tomoe Shimazaki,[1] Kazumasa Hiroishi,[1] Yuzo Miyakawa,[2] and Michio Imawari[1]

[1]*Department of Gastroenterology, Showa University School of Medicine, Tokyo, Japan*
[2]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Infection with hepatitis C virus (HCV) is associated with lymphoproliferative disorders, represented by essential mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, but the pathogenic mechanism remains obscure. HCV may infect B cells or interact with their cell surface receptors, and induce lymphoproliferation. The influence of HCV infection of B cells on the development of lymphoproliferative disorders was evaluated in 75 patients with persistent HCV infection. HCV infection was more prevalent (63% vs. 16%, 14%, or 17% $P < 0.05$ for each), and HCV RNA levels were higher (3.35 ± 3.85 vs. 1.75 ± 2.52, 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each) in B cells than $CD4^+$, $CD8^+$ T cells or other cells. Negative-strand HCV RNA, as a marker of viral replication, was detected in B cells from four of the 75 (5%) patients. Markers for lymphoproliferative disorders were more frequent in the 50 patients with chronic hepatitis C than the 32 with chronic hepatitis B, including cryoglobulinemia (26% vs. 0%, $P < 0.001$), low CH_{50} levels (48% vs. 3%, $P = 0.012$), and the clonality of B cells (12% vs. 0%, $P < 0.01$). By multivariate analysis, HCV RNA in B cells was an independent factor associated with the presence of at least one marker for lymphoproliferation (odds ratio: 1.98 [95% confidence interval: 1.36–7.24], $P = 0.027$). Based on the results obtained, the infection of B cells with HCV would play an important role in the development of lymphoproliferative disorders. ***J. Med. Virol. 81:619–627, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: B cells; clonality; cryoglobulinemia; hepatitis C virus; lymphoproliferative disorders

INTRODUCTION

Hepatitis C virus (HCV) can induce chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [Tong et al., 1995; Ikeda et al., 1998]. In addition, some patients infected with HCV develop proliferative disorders of lymphocytes, such as mixed cryoglobulinemia [Agnello et al., 1992; Frangeul et al., 1996; Donada et al., 1998] and B-cell non-Hodgkin's lymphoma (NHL) [Ferri et al., 1994]. Cryoglobulinemia represents the oligoclonal proliferation of B cells and occurs in 19–56% of patients infected with HCV [Mazzaro et al., 1996; Donada et al., 1998; Weiner et al., 1998; Schmidt et al., 2000], while antibody to HCV (anti-HCV) and HCV RNA are detected more frequently in the patients with non-Hodgkin's lymphoma than in the general population (30% vs. 1.3%) [Ferri et al., 1994]. On the basis of these observations, cryoglobulinemia is considered to be a marker for lymphoproliferative disorders. In addition, rheumatoid factor (RF) in high titers and hypocomplementemia (low levels of C3, C4, or CH_{50}) are regarded as immunological markers for autoimmune disease and lymphoproliferation [Ramos-Casals et al., 2005]. In the patients with Sjögren's syndrome, for instance, hypocomplementemia was closely associated with the development of lymphoma [Ramos-Casals et al., 2005].

Although an epidemiological association has been noted between HCV infection and lymphoproliferative disorders, the pathogenic mechanisms underlying it have remained unclear. HCV would infect B cells persistently, and induce somatic mutations toward propagation in them. Recently, the replication of HCV was demonstrated in a B-cell line established from a patient infected with HCV [Sung et al., 2003], and

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*Correspondence to: Takayoshi Ito, MD, Department of Gastroenterology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan.
E-mail: tito@med.showa-u.ac.jp

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somatic hypermutations in the immunoglobulin genes as well as proto-oncogenes were observed in a B-cell line infected with HCV [Machida et al., 2004]. These observations suggest direct and/or indirect effects of HCV infection of B cells on the induction of lymphoproliferative disorders. HCV infection by itself or stimulation by immune complexes containing viral antigens may trigger the clonal proliferation of B cells for the development of lymphoproliferative disorders in patients with chronic hepatitis C [Agnello, 1995; Ivanovski et al., 1998].

There is some evidence for the replication of HCV in peripheral blood mononuclear cells (PBMCs) of patients who are infected with HCV persistently [Moldvay et al., 1994; Lerat et al., 1998; Zignego and Brechot, 1999], although it is not known which of the T-, B-, and other cell-types supports the replication of HCV. It may replicate at very low levels in lymphoid cells, and they may serve as an extra-hepatic reservoir; this is implicated in recurrence and persistence of HCV infection in immunosuppressed individuals [Laskus et al., 2000]. Although current antiviral therapies can eliminate HCV from the serum, it may survive in lymphocytes thereafter [Pham et al., 2004; Radkowski et al., 2005]. The efficacy of interferon therapy on the HCV infection of lymphocytes is, as yet, uncertain.

The frequency of HCV infection of peripheral blood mononuclear cells (PBMCs) was examined in 75 patients with chronic hepatitis C, and HCV RNA levels were determined in $CD4^+$, $CD8^+$ T-cells, B-cells, and other cells. The replication of HCV in B cells was correlated subsequently with lymphoproliferative disorders.

MATERIALS AND METHODS

Patients

During 2003 through 2006, 75 patients infected with HCV were admitted to the Showa University Hospital, including two with acute hepatitis, 50 with chronic hepatitis, two with liver cirrhosis, 16 with hepatocellular carcinoma and five with non-Hodgkin's lymphoma. The diagnosis of HCV infection was confirmed in all these patients by the detection of anti-HCV and HCV RNA in the serum. All the patients were negative for hepatitis B surface antigen (HBsAg) or antibody to human immunodeficiency virus type-1. Serving as controls were 28 healthy individuals without HCV infection and 32 patients with chronic hepatitis B who did not have liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Informed consent was obtained from every participant for the purpose of this study, and the study was approved by the Ethics Committee of Showa University School of Medicine.

Isolation of Lymphoid Cells

PBMCs ($2.83 \pm 1.46 \times 10^7$) were obtained from whole blood (30 ml) by the centrifugation in a Ficoll/Hypaque gradient. Beads with the affinity for $CD8^+$ cells (MicroBeads®; Miltenyi Biotte, Bergisch Gladbach, Germany) were added to PBMCs, and the cell suspension was mixed well, incubated for 15 min at 4°C and centrifuged at 900*g* for 10 min in a tube. The tube was then placed on a magnet, and the supernatant floating free cells was transferred to another tube. The pellet containing $CD8^+$ cells was collected and stored at −80°C until use. $CD4^+$ and $CD19^+$ cells were separated from the supernatant using similar procedures. The remaining supernatant was pelleted to make the 'other cell' fraction. Each compartment of PBMCs contained $\sim 11 \times 10^5 - 10^6$ cells.

Quantitation of HCV RNA in Lymphoid Cells

HCV RNA was determined by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from each cellular compartment using Trizol® Reagent (Invitrogen, Carlsbad, CA). The RNA solution was stirred-up, and a portion (1 μl from the total of 50 μl) was reverse-transcribed by AMV® RT (Roche, Mannheim, Germany) and amplified by the single-step PCR for 40 cycles with appropriate primers (5′-CGC GCG ACT AGG AAG ACT TC-3′ and 5′-ATA GAG AAA GAG CAAC CA GG-3′) that are complementary to the 5′-UTR sequence. HCV RNA was determined in 100 ng of each RNA sample by the real-time RT-PCR using the primers described previously [Ito et al., 2001]; it has a detection range over 1.0–8.0 log copies. HCV RNA was recorded as positive for the samples with titers exceeding 1.0 log copies/100 ng, in order to exclude contamination of lymphoid cells with serum HCV RNA.

RT-PCR for Detecting Negative-Strand HCV RNA

Negative-strand HCV RNA was determined by the strand-specific RT-PCR. A portion of total RNA (1 μl from 50 μl) extracted from the B-cell fraction (10^5 cells) was reverse-transcribed with the sense primer (5′-AGA CAT CGG GCC AGA AGT GTC C-3′) complementary to a partial NS5B sequence of negative-strand HCV RNA, and amplified by the hot-started single-step RT-PCR for 40 cycles using the GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems, Branchburg, CA) with the same primer and an antisense primer (5′-CGT TCA TCG GTT GGG GAG CAG G-3′) located downstream of it [Castillo et al., 2005]. Controls were negative- and positive-strand HCV RNA species that had been generated by in vitro transcription using the pCVJ4L6S plasmid [Yanagi et al., 1998]. Assays were performed in duplicate for each sample.

Serum Markers of Lymphoproliferative Disorders

Cryoglobulinemia was detected by a semi-quantitative centrifugation method. Briefly, blood samples were centrifuged at 600*g* for 20 min at 37°C. Sera were cooled to 4°C and left to stand for 48 h, and centrifuged again at 2,500*g* for 10 min at 4°C. The emergence of cryocrit at 4°C and its disappearance by warming up to 37°C for

20 min was regarded positive for cryoglobulin. RF was determined by the latex turbidimetric assay, and C4 and CH_{50} activities by nephelometry and Mayer's method, respectively. Markers of lymphoproliferative disorders were determined in 50 patients with chronic hepatitis C and 32 patients with chronic hepatitis B who had HBsAg in the serum. None of the patients with chronic hepatitis suffered from acute hepatitis, cirrhosis or non-Hodgkin's lymphoma.

Amplification of the V_H Region in Immunoglobulin by PCR

RNA (1 μl) from PBMCs or B cells was reverse-transcribed into cDNA and amplified using the GeneAmp® EZ rTth RNA PCR kit (Applied Biosystems) in accordance with the manufacturer's instructions. Amplification was carried out with FW1 primer (5′-AGG TGC AGC TGG A[T]GG[C] AGT C[G]T[G]G G-3′) in the V_H region and hM3 primer (5′-GGA AAA GGG TTG GGG CGG AT-3′) located 8 nt downstream from the start of C_H1 exon in Cμ region. PCR products were visualized by staining with ethidium bromide after they had been electrophoresed on 1% agarose gels.

Fingerprinting Assay for the Ig Gene

The clonality of B cells was examined by the fingerprinting assay specific for isotypes of the immunoglobulin (Ig) gene by the method of Ivanovski et al. [1998]. Briefly, the PCR products of the Ig-V_H gene, from 75 patients infected with HCV and 28 healthy controls, were examined for the length of the complementarity-determining region 3 (CDR3) by primer extension using a primer (hFW3; 5′-CTG AGG ACA CGG CCG TGT ATT ACT G-3′) complementary to a conserved sequence in human V_H framework 3 (FW3) regions. The hFW3 primer was end-labeled with [γ-^{32}P]ATP (150 mCi/ml; Japan Radioisotope Association, Tokyo, Japan) using the phage T4 polynucleotide kinase (T4PNK; Takara, Shiga, Japan). The reaction mixture (18 μl), containing PCR products, 1 μM ^{32}P-labeled primer, 25 mM dNTP, 10× PCR buffer and 1 unit of Taq polymerase, was subjected to denaturation at 95°C for 8 min, annealing at 64°C for 1 min and extension at 72°C for 15 min. The reactant (9 μl) was separated by electrophoresis on 6% polyacrylamide gel supplemented with 6 M urea, dried and exposed onto radiographic films.

Subcloning and Sequencing of the V_H Region Gene

One PCR product from a patient infected with HCV that exhibited oligoclonal bands and another from a control subject, without displaying any clonal band on the Ig fingerprinting assay, were purified on gel-electrophoresis and sub-cloned into the pCR-TOPO vector (Invitrogen, Leek, The Netherlands). After they had transformed *Escherichia coli* (DH5α), 26 and 16 clones from the patient and control, respectively, were selected and the V_H region was sequenced using the Big Dye® Terminator ver.1.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan). Deduced amino acid sequences of the VDJ region of IgM heavy chain were aligned by the BLAST search, and analyzed for any homology among clones by the GENETYX-Mac ver.13.0 software (Genetyx, Tokyo, Japan).

Statistical Analysis

The median of continuous variables, without the normal distribution, was compared by the Mann–Whitney *U* test. Comparison of discontinuous variables was performed by the χ^2 test or Fisher's exact test. A P value < 0.05 was considered statistically significant. Values with the normal distribution were expressed as the mean ± SD. Data of variables, not distributed normally, were transformed into log values as required. Logistic regression modeling was used in the multivariate analysis for association with lymphoproliferative disorders with the JMP ver. 7 software (SAS Institute, Cary, NC).

RESULTS

Detection of HCV RNA in B Cells

Table I compares the detection of HCV RNA in various cellular compartments. HCV RNA was detected more frequently in B cells than $CD4^+$, $CD8^+$ T cells or other cells from the 75 patients (63% vs. 16%, 14%, or 17%, $P < 0.05$ for each). There were no significant differences in the detection of HCV RNA in lymphoid cells between the patients with chronic hepatitis and those with hepatocellular carcinoma or non-Hodgkin's lymphoma. HCV RNA was detected in B cells from 55 (73%) patients by the conventional RT-PCR at screening. Eight of them, with HCV RNA titers <1.0 log copies/100 ng RNA by the real-time RT-PCR, were deemed negative for HCV RNA

TABLE I. Frequency of HCV RNA in Diverse Cell Compartments From Patients Infected With HCV

Cell types	Total (n = 75)	Chronic hepatitis[a] (n = 54)	Non-Hodgkin lymphoma (n = 5)	Hepatocellular carcinoma (n = 16)
$CD8^+$ T cells	12 (16%)*	6 (11%)*	2 (40%)	4 (25%)
$CD4^+$ T cells	11 (15%)*	6 (11%)*	3 (60%)	2 (13%)
B cells	47 (63%)	32 (59%)	3 (60%)	12 (75%)
Others	17 (23%)*	11 (20%)**	3 (60%)	3 (19%)

[a]Two patients each with acute hepatitis and cirrhosis without hepatocellular carcinoma were included.
*Significantly lower than the detection in B cells ($P < 0.05$).
**Significantly lower than the detection in B cells ($P < 0.01$).

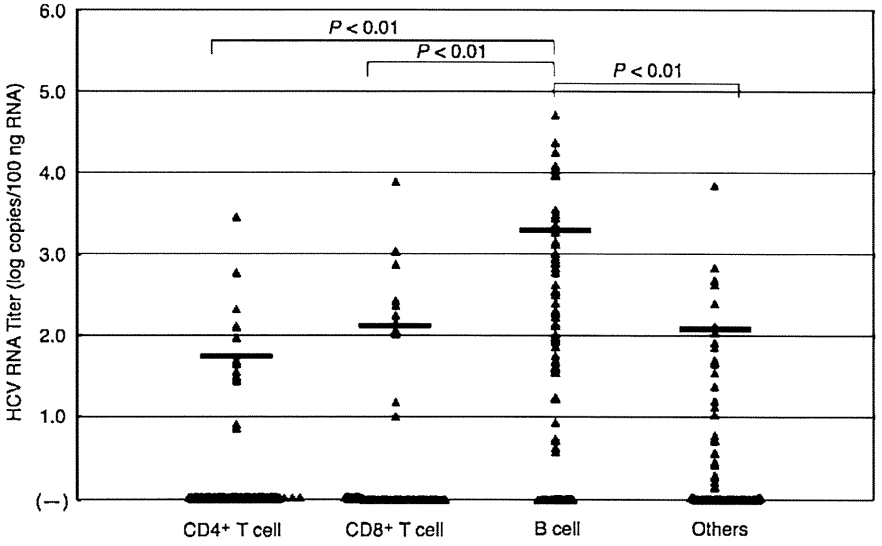

Fig. 1. HCV RNA titers in various compartments of lymphoid cells. Bold bars indicate mean values.

in order to exclude contamination by serum HCV RNA. The distribution of HCV RNA titers in various cell compartments is illustrated in Figure 1. HCV RNA levels were higher in B cells than in $CD4^+$, $CD8^+$ T cells or other cells (3.35 ± 3.85 vs. 1.75 ± 2.52, 2.15 ± 2.94 or 2.10 ± 2.90 log copies/100 ng, $P < 0.01$ for each).

Replication of HCV in B Cells

A method for detecting minus-strand HCV RNA was developed (see Materials and Methods Section). It could detect negative-strand HCV RNA specifically with a sensitivity of 3.0 log copies/ml (range: 3.0–6.0 log copies/ml) without a self-priming of positive strands in control transcripts (Fig. 2A). None of the 75 patients had >7.0 log copies of HCV RNA in B cells (Fig. 1), thereby indicating the capability of this method to detect negative-strand HCV RNA in the patients studied. Since this assay could not detect <3.0 log copies/ml of negative-strand HCV RNA, which were present in lower titers than positive strands usually, only the 16 patients with HCV RNA in B cells in titers ≥3.0 log copies/ml were analyzed. Negative-strand HCV RNA was detected in four (5%) of the 75 patients, indicating that HCV replicated efficiently in the B cells (lanes 2, 14, 15, and 17 in Fig. 2B).

Markers for Lymphoproliferative Disorders in Patients With Chronic Viral Hepatitis

Table II compares the detection of markers for lymphoproliferative disorders between the 50 patients with chronic hepatitis C and the 32 patients with chronic hepatitis; they did not have acute hepatitis, liver cirrhosis, hepatocellular carcinoma or non-Hodgkin's lymphoma. Cryoglobulinemia and low CH_{50} levels

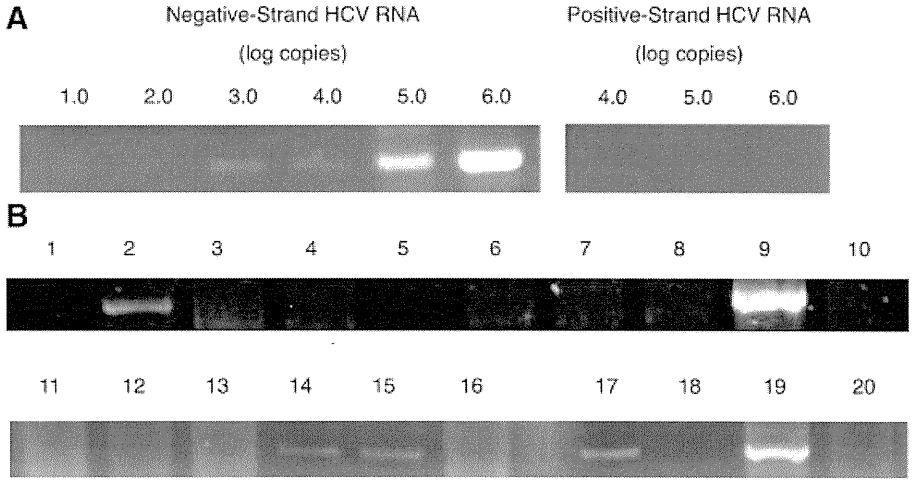

Fig. 2. **A**: Ethidium bromide staining of products of strand-specific RT-PCR for negative- and positive-strand HCV RNA. **B**: Strand specific RT-PCR for negative-strand HCV RNA from B cells of 16 patients. Positive control for negative-strand HCV RNA (**lanes 9** and **19**) and negative control for positive-strands HCV RNA (**lanes 10** and **20**) are included.

TABLE II. Markers for Lymphoproliferative Disorders in Patients Chronic Hepatitis C or B

Features[a]	Hepatitis C (n = 50)	Hepatitis B (n = 32)	Differences *P* value
Age (years)	52.3 ± 11.8	53.9 ± 11.9	NS
Men	32 (64%)	17 (53%)	NS
ALT (IU/L [5–25][b])	96.5 ± 145.9	50.7 ± 79.8	0.008
Cryoglobuminemia	13 (26%)	0 (0%)	<0.001
RF > 10 IU/ml	24 (48%)	13 (41%)	NS
C4 < 10 mg/dl	1 (2%)	1 (3%)	NS
CH_{50} < 20 U/ml	24 (48%)	1 (3%)	0.012
Clonality	6 (12%)	0 (0%)	<0.001
Any marker for lymphoproliferative disorders	37 (74%)	13 (41%)	0.015

ALT, alanine aminotranferase; RF, rheumatoid factor; NS, not significant.
[a]Data are number (%) or the mean ± SD.
[b]Interquartile normal range.

(<20 U/ml) were significantly more frequent in the patients with chronic hepatitis C than hepatitis B, while high RF titers (>10 IU/ml) were common in them both. At least one marker for lymphoproliferative disorders was detected more frequently in patients with chronic hepatitis C than hepatitis B (74% vs. 41%, $P = 0.015$). These results indicate that cryoglobulinemia and low CH_{50} levels would be markers of lymphoproliferative disorders characteristic of the patients with chronic hepatitis C.

Oligoclonality of the Immunoglobulin Heavy Chain (Ig-V_H) Gene in Patients With HCV Infection

Fingerprinting assay of the Ig-V_H gene was performed on B cells recovered from patients infected with HCV. Ladders of PCR products from B cells of healthy controls did not produce strong bands (Fig. 3A). In contrast, the ladders of some patients with HCV infection contained a monoclonal band indicative of the oligoclonality

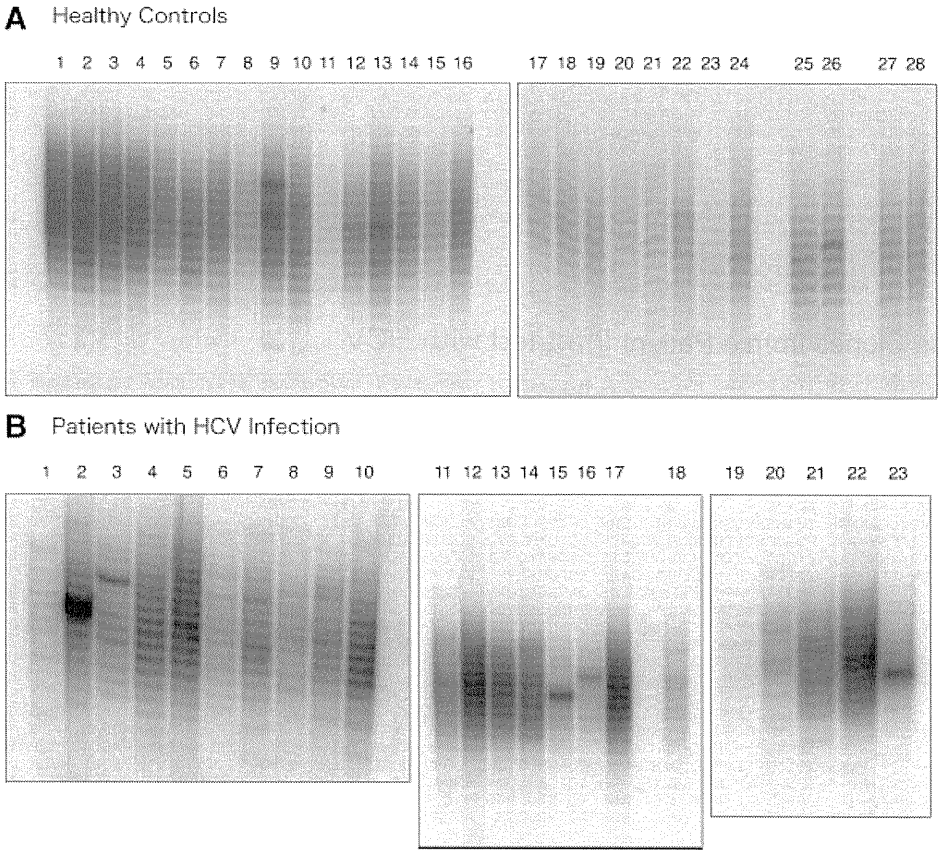

Fig. 3. Ig fingerprinting assay of (**A**) PBMCs from 28 healthy subjects and (**B**) B cells from 23 patients infected with HCV.

(Fig. 3B). The oligoclonality was observed not only in the patient with non-Hodgkin's lymphoma (lane 2 in Fig. 3B), but also in those without non-Hodgkin's lymphoma (lanes 3, 15, 16, and 23 in Fig. 3B). Overall, it was detected in B cells from 8 (11%) of the 75 patients infected with HCV, including one each with non-Hodgkin's lymphoma and hepatocellular carcinoma and six with chronic hepatitis. Six of these eight patients had B cells carrying HCV RNA, and seven possessed at least one of cryoglobulinemia (detected in three patients), high-titered RF (four patients) and hypocomplementemia (four patients). Six patients were infected with HCV group 1 and the remaining two with HCV group 2. When the same assay was undertaken on PBMCs from 32 patients with chronic hepatitis B, the oligoclonality was not observed in any of the patients (Table II).

Amino acid sequences of some clones, obtained from a patient whose Ig-V_H gene showed the oligoclonality (lane 3 in Fig. 3B), were similar (Fig. 4B). In contrast, clones obtained from a healthy subject without strong bands in Ig-V_H fingerprinting assay (lane 8 in Fig. 3A) were not similar in the sequence (Fig. 4A). These results suggest that a clonal expansion of B cells would occur in the peripheral blood of patients infected with HCV.

Factors Associated With Lymphoproliferative Disorders

Univariate analysis was performed to determine factors predisposing to lymphoproliferative disorders, including cryoglobulinemia, high-titered RF, hypocomplementemia and the B-cell clonality (Table III). Low ALT levels and the presence of HCV RNA in B cells were associated significantly with lymphoproliferative disorders. Among them, HCV RNA in B cells was the only predictive factor by multivariate analysis (odds ratio 1.98 [95% confidence interval 1.36–7.24], $P = 0.027$).

DISCUSSION

It has been demonstrated epidemiologically that HCV induces a number of extrahepatic manifestations [Cacoub et al., 1999; Zignego and Brechot, 1999], of which lymphoproliferative disorders is related most closely to HCV infection [Zignego et al., 2007]. Accordingly, it has been accepted that chronic infection with HCV can lead to the clonal expansion of B cells and that the sustained proliferation of B cells would promote the occurrence of genetic mutations. Zignego et al. [2000] have observed frequently t(14;18) translocation and overexpression of bcl-2 in lymphoid cells from patients with lymphoproliferative disorders in association with

(A) IgVH Gene Clones from a Healthy Control

```
C3   GDGDRGPLAP DVHTVVGTWA AILQRTVIHG RVVGSQATQL HVGCARGCVC GHRDSALELL CIVCVTIVSA DPSHPLKPLP RGLPHPADSI AGKGVTRSPA GDLH
C1   -------F-- ---V--VVGV -ATTT-T-TS T-IHGCVLGF QAVHLQIGDA FGII-*DGES AFHRRGVFCC PTISFASNET YP-QPLPW-L -NPAHSIITK -ESR
C2   ----QSS--- G-EP-YPLRV VPPHLSRTVI HGRVRSGHRA RLQGELALGR VY*YGDSTLE GG-V-GAPTV IYIPNPLQSL PWGLLDPAPV VTTDGATRDS EGEG
C13  ----Q-S--- V-KV-WASCC YSSTVIHGCV LGFQAVHL-I GDAFGIIS*D -ESA-HRRGV FCCPTISFAS NETY--Q--- WS-AD--H-- IT--EP--CT -ESQ
C10  -------P-- ---I--RPEP VNPTPDFCTL IEG-ALSREA AHL*IQ-VFA VVSGDGESA- HRV-IECCST I-TNKGD-LQ -LPWSLTYPM HGIIFKCESR -CTG
C7   ----Q-S--- V-NRGGK*FQ FGTVIHGRVL GSQAVHLQIQ *-LGVVSGDG ESALHRVCVV --TTTT-NV* N-LQ--PWSL ADPA-VVVTE GESRGCTGES QGPS
C12  R---HC---- -IKSIR*LPE P*KAG-RTVI HSRVLVSQAV -LLIQ*VLGI VSGDGESALH RVCV-YGTTT TTNV*NPLQ- LPWSLADPVH -IATEGE-RG CTGE
C16  -------F-- ---V-*ASQN IVSS----RS --L----VH- QIQ*VL-VVS -DGE---H*V -V-Y--TTTT NG*D--Q--- WS-ADPVHA- -TE-ESRGCT -ESQ
C14  ---VQ----- V-K-*PQST- -AARSNRTVI HGRVLGSQAV -LQIQ-VLGI ISG-GEPA-H GVCI-CATTT AINN*DPLQ- LPWSLADPAH GIAAKGESRG CTGE
C5   ----Q-S--- QP*ISRTVIH GCVV-SQPAQ LHV-CADRRV PGHGDPALKL LCIVCATTVR VDPSHPLKPL SRGLSHPVHI VAGEDVSRSL ---DLH
C4   ----H-XPG- SSQSKQLALX HXFSHSNTRP CPRLSGCSFA DTVSSWR-XW RW*IGPSQSX RSL-NYYYX* CMKPTPA-SL EPXGSSSCS- H*R*IQRLHR R-X-Q
C6   PPAAPKGEFA AAKFNSPYSE SYYNSLAVVL QRRDWENPGV TQLNRLAAHP PFASWRNSEE ARTDRPSQQL RSLYVRQFKV YTYKRESRYR LFVDVQSDII DTPGR
C8   MQPPNISLIS LAVKRIICYI **KVNTPTKT RRFSN*TF*S T*LYMAYRTY ALNIRLYAVP VNGMPHL*TK EFSPDEMGVF RYPPSQGLWQ EYGLLL*CQT APAAP
C11  SRATVTSAKT AR*STTCTYS R*RSPERARA IGISTT*CRR CPENPSTARF PFCKLLQSSI ILCRNQQGQA EDCIAHTCAS VDDTRMARTF STLRQLVVAV ICPAS
C9   -W-G*LQDKT *SRKRKYL*S KHFT*LSFCI YKL-IGREEK MGRGG**GS* LEVGSQTGIT LKT*RSRNRG KQTSARWQNR LF*CFSARLH QLH
C15  ETVTRAPWPQ *SND--APRS KGCLAQ*YTA VS-AVTELSF R-NWFLDVST DMVTRL-REG L*LVLAL**I PIHSSPFPGA AGSSSSSNY* WSHQGQC-RG T-SPK
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(B) IgVH Gene Clones from a Patient Infected with HCV

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HC17 GDGDRGPLAP DVHTVVGTTT IYGCRTVIHG RVLGSQATQL HVGCAYGHVP GNGDSFLEVL CVVCVITAND DPSYPLKALS TSLSHPLHSR TSKGEYRSLA GDLH
HC5  ---------- ---------- ---------S ---------- ---------- ---------- ---------- ---------- ---------- -----S---- ----
HC10 ---------- ---------- ---------- ---------- -----C---- ---------- ---------- V--------- ---------- -----S-G-- ----
HC16 ---V------ --------A- ----H----- ---------- -----C---- ---------- ---------- --------M- ---------- -----S---- ----
HC8  ---------- ---------- -H-------- ---------- -----C---- -----L--L- -----T---- ---------- ---------- AT---S---- ----
HC15 ---------- -------S-- -*G------- -------A-- -----C---L --------L- ------T--- ---------- ---------- G----S---- ----
HC3  ---------- ---------- -*----I-R- -------A-- ----PC---- T-G-----L- -----A---- G----P---- --PP------ G----S---- ----
HC24 ----Q-S--- V-K-FLNSIV ATI*C----- ---R---A-- ----VCRC-L G----A-L-- -I---TIVSA ---H--*P-- RG---SADTI AG--VT---- ----
HC7  ----Q-S--- VPYLQAANSR TVIHSRVLSS QAVHL-IQRV LGIVSGDGES ALHGVCVVFI TSII*YNCHP LQPLAWSLAD PVH-IASEGE SRGCTGESQG PPRL
HC26 R---HC---- -IKSSVAP-S PQLCSRTVIH SRVLVSQAVH LQIQ*GLGIV SGDGESALHR VC-VYG-TTT TNV*NPLQAL PWSLADPVHI ATEGESRGCG ESQ-
HC18 ----Q-SWPQ *SK*SNQPLL Y*SLAQ*YTA VSAAVTELSF RENWFLDVST DMVSRL-REG L*LVLPL**I YPIHSSPFPG GCRVQLQ**L LMEPPETVQV RGRV
HC4  --S-Q-AT-- ETEVP-VAA- TTTPPISRTV IYSRVCGAH* AQPQGELV-G RVYVCSDSS- EGRV-EG-PT SKHTPNPLQP LLWGLADPGP VVVTTDDGVT KDSA
HC12 ---------- ------VVRI RDNSPNHMFS T-IYGRVLGS QAVHLQIQRV LGIVSGDGKP ALHG-SVVCV TTTSIN**DP LQPLPWSPAD PVHGIRAKGG SRGY
HC13 ---------- -----LPNRS H-DGGISRTV IHGRVLGSQA VHLQIL*VLG VVSGDGEPA- HGVCVVCTTT ATTNG*DPLQ PLPRSLADTT HGIVPKGESR _CTG
HC6  ----Q-S--- V-KGGVI--I -IVPTPKIPH T-IYGRVLGS QAVHLQIQRV LGI-PGDGES ALHG-CIVFI TSIIPYNCHP LQPLAWSLAD PVHAIATEGE SRRC
HC25 ----Q-S--- V-KVPRI-PR TLPISRTVIH SRVLGSQAVH LQIQRVLGIV SGDGEWALHG VCIVFITSII *YNCHPLQPL AWSLADPVHA IATEGESRGC TGES
HC22 --S-Q-AT-- VTEVPPI-SR C-CRTVIHSR VLDSHAVHLQ IQRVLELSLE MVNRPFA-SA *YLSLPSFHI TATHSSPSPG AWRTQCMP*L PKVDPDAAQD SLRD
HC19 ----Q-S--L G-EPAGS-LF RTVIHSRVL- SQVVHLQI-R VL-IVS-DGE SALHGVCI-F ITSIIPYNCH PLQPLAWS-A DPVHAIATQG E-RRFTGESQ _PPR
HC9  ----Q-S--- VPYLQAANSR TVIHSR-LSS QAVHL-IQRV LGIVSGDGES ALHGVCVVFI TSII*YNCHP LQPLAWSLAD PVH-IASEGE SRGCTGESQG PPRL
HC21 ----X-X-PQ ---X--VVFP HTIVEI*FSH SNRQPLSSG- RHVHLHI-AC YWSRLXXW*I GPSRSLHSSS YFHLLI*LPP -PAPCLEPGG HSACQNX*SV NAMR
HC2  ----Q-S--- VPQRNCSHLY FS-TVIHCRV LGSQAVHL-I Q*VLGVVSGD -ESALHRVHI VFLTSILLYV GHPLQPLPWP ADPAH-IATE GESRGCTGES QGPD
HC23 ----Q-S--- V-KMCSSYHH YHRISRTVIH GRALGSQAVH LHIQ*VLG-V SGDGESALHR VHIVFL-SIL LYVGHPLQPL PWSLADPAHP IATKGESRGC TGES
HC1  ---------- ------VVKV VQLPEPFLVS RTVIHSRVLG SQAVHLQIQR VLGVVSGDGE SALH*VSVVY VTTTTTNG*D PLQPL-WSLA DPVHAIATEG ESRG
HC20 R---HC---- -IKSS-AP-S PQLCSRTVIH SRVLVSQAVH LQIQ*GLGIV SGDGESALHR VCVVYG-TTT TNV*NPLQAL PWSLADPVHA IATEGESRGC TGES
HC14 ----Q-S--- V-NKAT---- -SPRTVIRSR VLGSQAVHLE IQ*VLGVVSG DAESALHIVC VIYATTLLLM YETHSSPFPG AWRTQFIS*L LKVHQRLHRR VSGT
HC11 ----Q-S--- V-NCTGWSFR TVI*GRVVDS ETVHL*IYSV LGIVSGDGE- ALHGVCVLCG ITMTNMCDPL QSPPWSLADP -HVRGTKGES RSCTGESQGP PG-Y
```

Fig. 4. Aligned amino acid sequences of (**A**) the 16 Ig-V_H gene clones from a healthy control (lane 8 in Fig. 3A) and (**B**) the 26 clones from a patient infected with HCV (lane 3 in Fig. 3B). Dashes indicate the sequence identity. Three clones from the patient with more than 95% homology are boxed.

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

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

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

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

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

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

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

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

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

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

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

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

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Original Article

Cost-effectiveness analysis on the surveillance for hepatocellular carcinoma in liver cirrhosis patients using contrast-enhanced ultrasonography

Hironori Tanaka,[1,2] Hiroko Iijima,[1,2] Kazuhiro Nouso,[3] Noriaki Aoki,[4] Takashi Iwai,[1] Tomoyuki Takashima,[1] Yoshiyuki Sakai,[1] Nobuhiro Aizawa,[1] Kazunari Iwata,[1] Naoto Ikeda,[1] Yoshinori Iwata,[1] Hirayuki Enomoto,[1] Masaki Saito,[1] Hiroyasu Imanishi[1] and Shuhei Nishiguchi[1]

[1]*Division of Hepatobiliary and Pancreatic Disease, and Department of Internal Medicine, and* [2]*Ultrasound Imaging Center, Hyogo College of Medicine, Nishinomiya, Hyogo,* [3]*Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan and* [4]*School of Health Information Sciences, University of Texas Health Science Center-Houston, Houston, USA*

Aim: Sonazoid is a new contrast agent for ultrasonography (US). Contrast-enhanced ultrasonography (CEUS) using Sonazoid enables Kupffer imaging, which improves the sensitivity of hepatocellular carcinoma (HCC) detection. However, there are no studies on the cost-effectiveness of HCC surveillance using Sonazoid.

Methods: We constructed a Markov model simulating the natural history of HCV-related liver cirrhosis (LC) patients, and compared three strategies (no surveillance, US surveillance and CEUS surveillance). The transition probability and cost data were obtained from published data. The simulation and analysis were performed using TreeAge pro 2009 software.

Results: When compared to the no surveillance group, the US and CEUS surveillance groups increased the life expectancy by 1.67 and 1.99 quality-adjusted life-years (QALY), respectively, and the incremental cost effectiveness ratio (ICER) were 17 296 $US/QALY and 18 384 $US/QALY, respectively. These results were both less than the commonly-accepted threshold of $US 50 000/QALY. Even if the CEUS surveillance group was compared with the US surveillance group, the ICER was $US 24 250 and thus cost-effective. Sensitivity analysis showed that the annual incidence of HCC and CEUS sensitivity were two critical parameters. However, when the annual incidence of HCC is more than 2% and/or the CEUS sensitivity is more than 80%, the ICER was also cost-effective.

Conclusions: Contrast-enhanced ultrasonography surveillance for HCC is a cost-effective strategy for LC patients and gains their longest additional life years, with similar degree of ICER in the US surveillance group. CEUS surveillance using Sonazoid is expected to be used not only in Japan, but also world-wide.

Key words: contrast-enhanced ultrasonography, cost-effective analysis, hepatocellular carcinoma, Sonazoid, surveillance

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common neoplasm in the world.[1] Although many environmental factors, including aflatoxins and alcohol,[2,3] have been implicated in the development of HCC, hepatitis B virus and hepatitis C virus (HCV) are the most important factors associated with the progression from chronic hepatitis to cirrhosis, and eventually to HCC.[4] Surveillance for HCC is recommended in patients with chronic liver injury to detect small-sized HCCs, which can be efficiently treated.[5] Ultrasonography (US) is a major surveillance method, because it provides low cost, real-time and non-invasive detection. However, there are some problems associated with this surveillance approach. It is known that the annual incidence of HCC increases with the degree of fibrosis.[6] Unfortunately, an increase in fibrosis makes US surveillance substantially more difficult, because the

Correspondence: Dr Hironori Tanaka, Division of Hepatobiliary and Pancreatic Disease, and Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. Email: hironori@hyo-med.ac.jp
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intrahepatic echo patterns in US become rough with advanced fibrosis.

Recently, a novel intravenous contrast medium for US, "Sonazoid", has become available in Japan. This strategy of using US with Sonazoid dramatically improves the sensitivity in the diagnosis of hepatic malignancy.[7] Thus, contrast-enhanced ultrasonography (CEUS) using Sonazoid can effectively detect HCCs that are usually overlooked by B-mode, which is currently used for observation. Therefore, this new contrast medium would be desirable for use in HCC surveillance. However, it is almost five times more expensive than the conventional observational approach in Japan. Until now, the surveillance for HCC using this novel agent has not been evaluated with regard to its cost-effectiveness, and this is the focus of the current study.

METHODS

WE USED TREE Age Pro 2009 (Tree Age Software Inc., William-stown, MA, USA) software to construct a Markov model, and estimated the cost-effectiveness of a surveillance program for HCC. The transition probabilities used in the analysis are listed in Table 1. The age specific mortality rate was obtained

Table 1 Values used in the analyses

Variable	Base value	Range	References
Excess annual mortality			
Child A Cirrhosis	0.02	0.00–0.08	8–11
Child B/C Cirrhosis	0.13	0.07–0.40	
Large HCC	0.90	0.50–1.00	12–14
Annual transition rate			
Child A to Child B/C	0.04	0.02–0.08	8,10,15,16
Small HCC to Large HCC (Undetected)*	0.30	0.10–0.60	17–19
Small HCC to large HCC (TAE treated)*	0.10	0.02–0.20	20–22
Annual incidence of HCC			
Incidence of new HCC	0.07	0.01–0.08	6,8,23–27
Incidence of HCC after curative treatment	0.20	0.10–0.37	13,25,28
Probability of small HCC at diagnosis	0.90	0.66–1.00	23,29
Test characteristics			
US			
Sensitivity	0.70	0.40–0.80	30–32
Specificity	0.90	0.70–0.90	
CEUS			
Sensitivity	0.90	0.80–0.95	7
Specificity	0.95	0.80–0.95	
Cost data			20,23,31,33–37
US	61		
CEUS	248		
Confirmation test	862	170–1 100	
LC	587	300–1 200	38
Decompensated LC	6 422	6 422–23 000	38
Terminal care	5 556	5 000–42 000	38
Resection	19 390	12 000–40 000	39
RFA	10 333	35 000–11 000	39
TAE	7 778	35 000–12 000	
Health-related QOL			40
Child A	0.75	0.66–0.83	
Child B/C	0.66	0.46–0.86	
HCC	0.64	0.44–0.86	

*Per 6 months. The costs were $US/6 months, and the baseline cost has been adjusted to US dollars (Currency rate: $1.00 = \90.00).
CEUS, contrast-enhanced ultrasonography; HCC, hepatocellular carcinoma; LC, liver cirrhosis; QOL, quality of life; RFA, radio-frequency ablation; TAE, transcatheter arterial embolization; US, ultrasonography.

from the homepage of the Japanese Ministry of Health, Labour, and Welfare.

Decision model

We estimated the long-term outcomes of different treatments by modifying a previously published computer simulation model[41] using current data on the natural history of chronic hepatitis C in Japan (Fig. 1). Each cycle consisted of 6 months. During each cycle, patients died according to the population-based mortality.

The decision tree for our analysis was composed of three arms: (i) the no surveillance group or "no surveillance" (ii) the B-mode US surveillance group or "US group", and (iii) the CEUS surveillance group or "CEUS group".

Assumptions 1 (program)

Based on the limited information available in the literature, the following assumptions were made:

1 the transition data from liver cirrhosis (LC) to decompensated LC are constant regardless of the patient's age and prior history of HCC;
2 the progression from compensated to decompensated cirrhosis is irreversible;
3 the incidence of HCC is the same in compensated versus decompensated cirrhosis.
4 the probabilities of HCC recurrence and growth remain constant over time;
5 surgery is not performed in patients with a background of decompensated cirrhosis or HCC recurrence; and
6 liver transplantation is not the first-choice for HCC therapy because it is still very rare in Japan.

Assumptions 2 (surveillance)

With regard to surveillance, the following assumptions were made:

1 HCC can be divided into two categories: "small" and "large". Small tumors (1–5 cm in diameter, and no

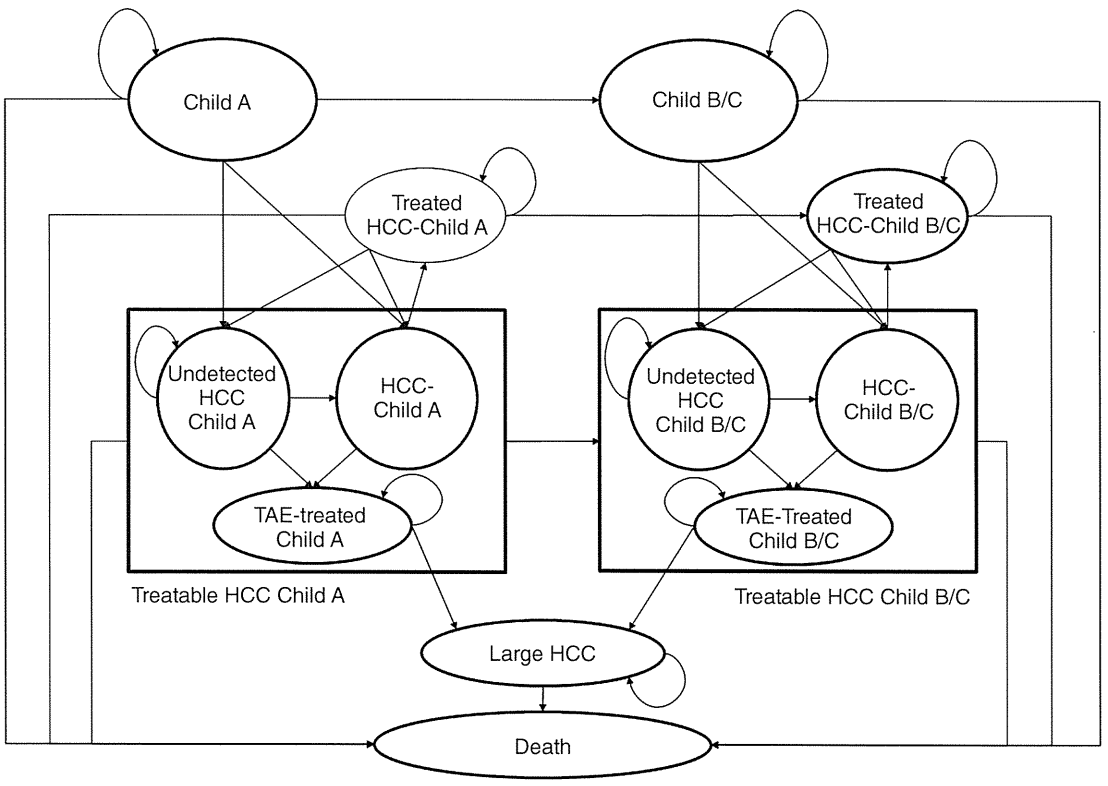

Figure 1 Natural history model. The arrows represent possible transitions during each 6 month cycle. Patients enter this model with Child A cirrhosis, and might develop Child B/C cirrhosis, hepatocellular carcinoma (HCC), both Child B/C and HCC, or death. If the health status does not change, then the patients remain in the same state of health. Surveillance and treatment strategies were superimposed on this model. TAE, transcatheter arterial embolization.

more than three in number) are asymptomatic, and remain undetected until the surveillance is performed. Large tumors are symptomatic, and the patient can receive palliative treatment only;
2 there are no small HCCs that can be detected incidentally in the no surveillance group;
3 patients with positive surveillance tests undergo a confirmatory test. [CT and either MRI (70%) or liver biopsy (30%)];
4 the test performance is independent of previous test results;
5 compliance with the program is 100%; and
6 there is a small rate of false-positive diagnoses, which will be discovered before any treatment.

The tumor growth rate was calculated with the assumption of a doubling time of 120 days.[17,18,42]

Since one year's worth is different in the health status, health-states utility should be taken into account for cost effectiveness analysis. Thus, we obtained the health-state utility information from meta-analysis.[40] The survival and costs were also discounted at the commonly accepted annual rate of 3%, because time and cost of distant future are generally thought to be of less value than those of present time.

Cost

The cost data shown in Table 1 are from data published in Japan, because Sonazoid is currently available only in Japan.

The data were converted to US currency at the exchange rate of US$1.00 = JP¥90.00. The cost of transcatheter arterial embolization (TAE) was estimated by including health insurance reimbursement using the reimbursement data in our hospital, because there were no available national data.

Sensitivity analysis

The results obtained from this model depended on the values that were used in the study; therefore a one-way sensitivity analysis was performed on all variables.

RESULTS

Accuracy of our model

TO VALIDATE THE model's accuracy, we compared this model's survival rate with the cumulative survival rates of 417 compensated cirrhosis patients obtained from a large European cohort clinical study under surveillance.[43] When we set the annual incidence rate of HCC as 4% to fit the European model, these two survival curves were very similar, and the accuracy of our model was validated (Fig. 2).

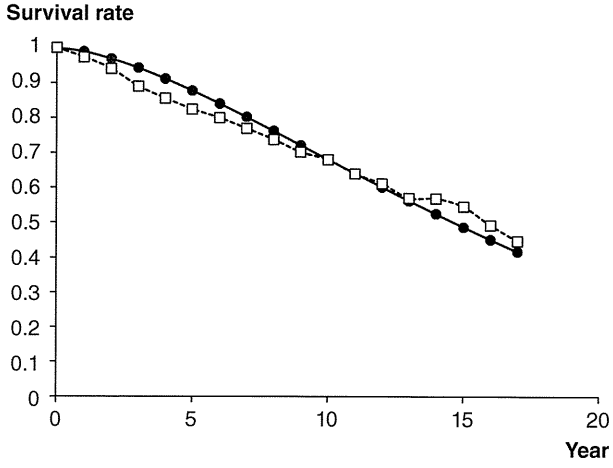

Figure 2 Comparison of the survival curves for compensated cirrhosis states between the one predicted by the current model and published data from a large cohort study.[43] Both data sources yielded similar curves. –●–, our model; -□-, Sangiovanni *et al.* 2004[43].

Baseline analysis

The expected life years of each group according to the starting age of the surveillance are shown in Figure 3.

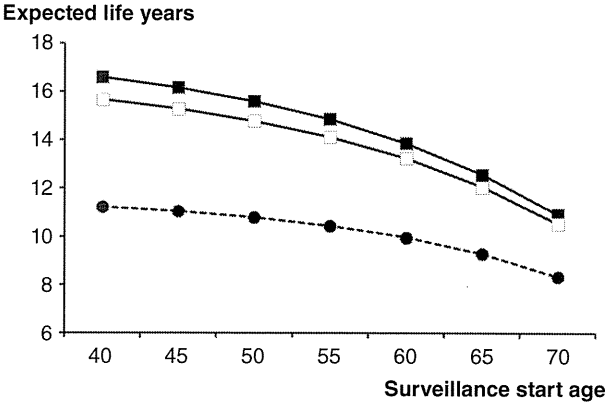

Figure 3 Expected life years according to surveillance at a starting age before it was discounted, and adjusted by health-state utilities. Although the expected life years decreased with age, both the ultrasonography (US) and contrast-enhanced ultrasonography (CEUS) surveillance groups increased the life expectancy even in 70-year-old patients. CEUS surveillance achieved the greatest gain in life expectancy in all analyzed age groups. –■–, CEUS Surveillance; –□–, US Surveillance; -●-, No Surveillance.