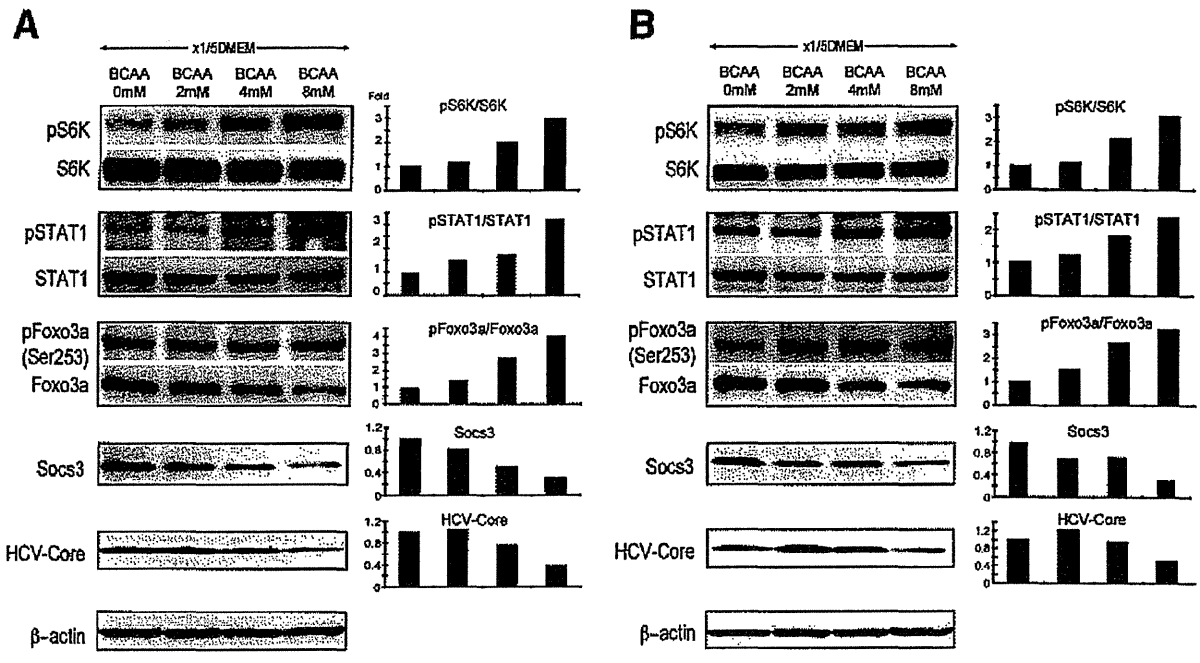

Figure 6. Expression of S6K, STAT1, Foxo3a, Socs3, and HCV core in H77S.3/GLuc2A-transfected Huh-7.5 cells or continuously JFH-1–infecting Huh-7 cells supplemented with BCAA.

ment of patients with liver malnutrition and impaired mTORC1 signaling would lead to reduced induction of ISGs. Importantly, BCAA was able to restore impaired IFN signaling through increased binding of ISGF3γ to its targets (Figure 2*D–F*).

Besides cross-talk of mTORC1 and IFN signaling, we revealed that Foxo3a also is involved in the IFN inhibitory pathway. In low-amino-acid medium, expression of pFoxo3a (ser253) was decreased substantially whereas that of Socs3 was increased. A decreased pFoxo3a/Foxo3a ratio indicates nuclear accumulation of Foxo3a before activation of its target genes, and this was confirmed by immunofluorescent staining (Figure 3*C*). The expression of Foxo3a was significantly positively correlated with that of Socs3 in CH-C liver (Figure 3*F*). These findings prompted us to identify a putative FBE in the Socs3 promoter region (Figure 4*A*). In fact, Socs3 promoter reporter activity was activated by overexpression of Foxo3a, and mutation of FBE impaired Foxo3a-dependent Socs3 promoter activation. Conversely, induction of Socs3 was not observed when expression of Foxo3a was knocked down by siRNA in low-amino-acid medium. Socs3 induction in low-amino-acid medium was owing to increased binding of Foxo3a to the FBE, which was confirmed by ChIP (Figure 4*D*). Therefore, in addition to impaired mTORC1 signaling, the Foxo3a-mediated Socs3 IFN inhibitory pathway might be involved in impaired IFN signaling in patients with liver malnutrition (Figure 4*E*).

Finally, we examined whether BCAA could restore impaired IFN signaling and inhibit HCV replication in cells under conditions of malnutrition. Importantly, BCAA could repress replication of the recombinant genotype 1a-derived HCV, H77S.3/GLuc2A, in a dose-dependent manner (Figure 5*A*). H77S.3/GLuc2A RNA produces infectious virus[14] and, therefore, the results indicate that BCAA might act on a naive HCV infection. Moreover, BCAA inhibited JFH-1–infected Huh-7 cells in which JFH-1 continuously was infecting in a dose-dependent manner. These results indicate that BCAA had an inhibitory effect on either naive or persistent HCV infection irrespective of genotypes (1a and 2a). Consistent with these results, BCAA induced the expression of pSTAT1 and Mx protein in a dose-dependent manner, and repressed Socs3 expression through increasing the ratio of pFoxo3a (ser243) to Foxo3a in a dose-dependent manner (Figures 5 and 6). Therefore, BCAA potentially could restore impaired IFN signaling and inhibit HCV replication in a CH-C state of malnutrition.

In conclusion, we addressed the clinical significance of the nutritional state of the liver on the treatment response of Peg-IFN and RBV combination therapy for CH-C. Although further studies are required to fully define the precise mechanisms underlying mTOR and IFN signaling, we showed that plasma values of Fischer's ratio are a useful nutritional parameter associated with treatment response. Fischer's ratio reflects mTORC1 signaling in the liver, which is correlated with IFN signaling and related to Socs3 IFN inhibitory signaling through Foxo3a. The potential usefulness of BCAA for the augmentation of IFN signaling could suggest a new therapeutic application for advanced-stage CH-C.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.03.051.

Appendix A

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Acknowledgments

Participating investigators from the Hokuriku Liver Study Group are listed in Appendix A.

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Conflicts of interest

The authors disclose no conflicts.

Supplementary Materials and Methods

Plasma Amino Acid Analysis

Plasma sample amino acid concentrations were measured by high-performance liquid chromatography–electrospray ionization–mass spectrometry followed by derivatization.[1] An MSQ Plus LC/MS system (Thermo Fischer Scientific, Waltham, MA) equipped with an electrospray ionization source was used in positive ionization mode for selected ion monitoring. Xcalibur version 1.4 SR1 software (Thermo Fischer Scientific, Yokohama, Japan) was used for data collection and processing. The high-performance liquid chromatography separation system consisted of an L-2100 pump, L-2200 autosampler, and L-2300 column oven (Hitachi High-Technologies Corporation, Tokyo, Japan). A Wakosil-II 3C8-100HG column (100, 2.1, 3 mm; Wako Pure Chemical Industries, Osaka, Japan) was used for the separation, and the mobile phase consisted of eluent A (25-mmol/L ammonium formate in water, pH 6.0) and eluent B (water:acetonitrile = 40:60).

Western Blotting

The expression of HCV core protein, Socs3, Foxo3a, phospho-Foxo3a (Ser253) (pFoxo3a), STAT1, pSTAT1 (Tyr701), S6K, pS6K, p-mTOR (Ser2448), Raptor, and β-actin were evaluated with mouse anti-core (Affinity BioReagents, Golden, CO), mouse anti-Socs3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Foxo3a, rabbit anti-β-actin (Sigma-Aldrich, St Louis, MO), rabbit anti-phospho-Foxo3a (Ser253), rabbit anti-STAT1, rabbit anti-p-STAT1 (Tyr701), rabbit anti-p70 S6K, rabbit anti-pS6K, rabbit anti-p-mTOR (Ser2448), and rabbit anti-Raptor (Cell Signaling Technology, Beverly, MA), respectively. Densitometric analysis was conducted directly on the blotted membrane using a charge coupled device camera system (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and Scion Image software (Frederick, MD).

Primer Sequences for PCR and siRNA

Primer sequences for PCR and siRNA were as follows: 2′5′OAS: forward 5′- CTC AGA AAT ACC CCA GCC AAA TC-3′, reverse 5′-GTG GTG AGA GGA CTG AGG AA-3′; Socs3: forward 5′-TAC CAC CTG AGT CTC CAG CTT CTC-3′, reverse 5′-CCT GGC AGT TCT CAT TAG TTC AGC ATT C-3′; Foxo3a: forward 5′-TGC TGT ATG CAA GAA CTT TCC AGT AGC AG-3′, reverse 5′-ACT CTA GCC CCC ATG CTA CTA GTG-3′; glyceraldehyde-3-phosphate dehydrogenase: forward 5′-GAA GGT GAA GGT CGG AGT-3′, reverse 5′-GAA GAT GGT GAT GGG ATT TC-3′, siFoxo3a (SASI_Hs01_00119127; Sigma) sense: 5′-GAA UGA UGG GCU GAC UGA AdTdT-3′, antisense: 5′-UUC AGU CAG CCC AUC AUU CdTdT-3′. Small interfering Raptor was purchased as part of KIAA1303 siGENOME SMART pool siRNA reagents from Dharmacon, Inc (Lafayette, CO).

Construction of ISRE-Luc Reporter and FBEmut-luc Reporter Plasmids

Oligonucleotides containing the ISRE tandem repeat sequence (sense 5′-TCG AGA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA ACT GAA A-3′, antisense 5′-AGC TTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT TCA GTT C-3′, consensus 5′-GAA Ann GAA ACT-3′) were annealed, and integrated into Xho I and Hind III sites of the pGL4.23 luciferase vector (Promega). The human Socs3 promoter region (-109/+217) was amplified by genomic PCR using specific primers (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG-3′ and reverse, 5′-CCG TGA AGT CCA CAA AGG AGC CTT C-3′) and cloned into the EcoR V site of the pGL4.10-luc2 reporter vector (Promega). The Socs3 FBE mutant reporter vector was created by substituting 2 adenines in the putative FBE with guanines (wild-type sequence 5′-CTAAACA-3′, mutated sequence 5′-CTGAGCA-3′).

ChIP Assay

For the ChIP assay using the anti-ISGF3γ antibody, 1×10^6 Huh-7 cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid medium for 6 hours. For ChIP using the anti-Foxo3a antibody, 1×10^5 Huh-7 cells were cultured in low-amino-acid medium for 24 hours.

Cells were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C, and the reaction was stopped with 250 mmol/L glycine for 10 minutes. Cells were suspended in sodium dodecyl sulfate–lysis buffer (1% sodium dodecyl sulfate, 10 mmol/L ethylenediaminetetraacetic acid [EDTA], 50 mmol/L Tris-HCl [pH 8.1]), complete protease inhibitor cocktail (Roche Applied Science), and incubated for 30 minutes at 10°C. Cell lysate was sonicated with Bioruptor (Cosmo Bio, Tokyo, Japan) to obtain chromatin fragments and diluted 10-fold in ChIP dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton-X 100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl, complete protease inhibitor cocktail). Chromatin fragments were incubated with 2 μg ISGF3γ antibody (Santa Cruz Biotechnology), 2 μg Foxo3a antibody (H-144; Santa Cruz Biotechnology), or normal rabbit immunoglobulin G for 18 hours at 4°C. Dynabeads (30 μL) protein G (Invitrogen) was added and incubated for 1 hour at 4°C. The beads were washed with low-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 150 mmol/L NaCl), high-salt-wash buffer (0.1% sodium dodecyl sulfate, 1% Triton-X 100, 2.0 mmol/L EDTA, 20 mmol/L Tris-HCl [pH 8.1], 500 mmol/L NaCl), LiCl wash buffer (250 mmol/L LiCl, 1% NP-40, 1% de-

oxycholate, 1.0 mmol/L EDTA, 1.0 mmol/L Tris-HCl [pH 8.1]) and Tris-EDTA buffer. Immunoprecipitated chromatin fragments were eluted with elution buffer (1% sodium dodecyl sulfate, 100 mmol/L NaHCO3, 10 mmol/L dithiothreitol), and reverse cross-linked by incubating for 6 hours at 65°C in elution buffer containing 200 mmol/L NaCl. DNA fragments were purified and quantified by real-time detection PCR with primers for putative ISRE in the 2′5′OAS promoter region (forward, 5′-AAA TGC ATT TCC AGA GCA GAG TTC AGA G-3′, reverse, 5′-GGG TAT TTC TGA GAT CCA TCA TTG ACA GG-3′) or putative FBE in the Socs3 promoter region (forward, 5′-TGC TGC GAG TAG TGA CTA AAC ATT ACA AG -3′, reverse, 5′-AGC GGA GCA GGG AGT CCA AGT C -3′). Values were normalized by the measurement of input DNA.

pH77S.3/GLuc2A

pH77S.2 is a modification of pH77S[2] containing an additional mutation within the E2 protein (N476D in the polyprotein) that promotes infectious virus yields from RNA-transfected cells (Yi et al, unpublished data). To monitor replication, the GLuc sequence, fused at its C terminus to the foot-and-mouth disease virus 2A autoprotease, was inserted between p7 and NS2 of pH77S.2 (Supplementary Figure 4). To insert the GLuc-coding sequence between p7 and NS2 in pH77S.2, followed by the foot-and-mouth disease virus 2A protein-coding sequence, Mlu I, EcoR V, and Spe I restriction sites were created between the p7 and NS2 coding sequences by site-directed mutagenesis. DNA coding for GLuc was subcloned into the Mlu I and EcoR V sites of the modified plasmid after PCR amplification using the primers: 5′- ATA ATA TTA CGC GTA *TGG GAG TCA AAG TTC TGT TTG CC*-3′ (sequence corresponding to the N-terminal GLuc is italicized and that corresponding to Mlu I is underlined) and 5′-ATA AAT AGAT ATC *GTC ACC ACC GGC CCC CTT GAT CTT*-3′ (C terminal GLuc is italicized and EcoR V is underlined). A DNA fragment encoding the 17 amino acids of the foot-and-mouth disease virus 2A protein was generated by annealing the following complementary oligonucleotides: 5′- ATA TGA TAT CAA CTT TGA CCT TCT CAA GTT GGC CGG CGA CGT CGA GTC CAA CCC AGG GCC CAC TAG CAT AT-3′ and 5′-ATA TGC TAG TGG GCC CTG GGT TGG ACT CGA CGT CGC CGG CCA ACT TGA GAA GGT CAA AGT TGA TAT CAT AT-3′ (underlined sequences indicate EcoR V and Spe I sites). The annealed oligonucleotides were digested by both restriction enzymes and the product inserted into the corresponding sites of pH77S.2 containing GLuc to generate pH77S.2/GLuc2A. Q41R is a cell-culture adaptive mutation within the NS3 protease domain of pH77S. Because it is not essential for production of infectious virus (Yi et al, unpublished data), pH77S.2 and pH77S.2/GLuc2A constructs underwent this mutation by site-directed mutagenesis of a PCR fragment spanning the Afe I and BsrG I sites to replace Gln_{41} with wild-type Arg. The resulting plasmids (pH77S.2/R41Q and pH77S.2/GLuc2A/R41Q) were redesignated pH77S.3 and pH77S.3/GLuc2A, respectively.[3,4] GLuc has several advantages over other luciferase reporter enzymes in that it is smaller and allows more sensitive detection than either firefly or Renilla luciferase.[3,4] In addition, a signal sequence directs its secretion into cell-culture media, allowing real-time dynamic measurements of GLuc expression without the need for cell lysis. H77S.3/GLuc2A RNA produces infectious virus, although with lower efficiency than H77S.3 RNA (10-fold less).

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Frequency of CD45RO$^+$ subset in CD4$^+$CD25^{high} regulatory T cells associated with progression of hepatocellular carcinoma

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ABSTRACT

The purpose of this study was to assess the properties of CD4$^+$CD25$^{high/low/negative}$ T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of CD45RO$^+$ cells in CD4$^+$CD25^{high} T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the CD45RO$^+$ subset in CD4$^+$CD25^{high} Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1–3].

CD4$^+$CD25^{high} Foxp3$^+$ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4–6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients with several different tumor types [3–12]. It has been reported that, in HCC patients, increased Tregs are correlated with CD8$^+$ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8,13–15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation [16,17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be

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implicated in the regulation of immune responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF-β1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of $CD4^+CD25^{high}$ $Foxp3^+$ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than $CD4^+CD25^{low/negative}$ cells. Furthermore, the proportion of $CD45RO^+$ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and healthy controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography (CT), magnetic resonance imaging (MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

Table 1
Clinical characteristics of hepatocellular carcinoma, liver cirrhosis, chronic hepatitis patients and healthy control.

Hepatocellular carcinoma (n = 62)	
Age (yrs)	68.9 ± 9.5
Gender (M/F)	37/25
Etiology of liver disease	
HBV/HCV/HBV + HCV/NBNC	19/34/2/7
TNM stages I/II/III/IV-A/IV-B	18/12/20/6/6
Largest tumor (mm)	
Child-Pugh A/B/C	37.6 ± 34.4
AFP (ng/mL)	41/8/3
DCP (mAU/mL)	10–35,093 (52)
Liver cirrhosis (n = 16)	
Age (yrs)	10–32,818 (34)
Gender (M/F)	58.3 ± 10.3
Etiology of liver disease	11/5
HBV/HCV/NBNC	4/7/5
Chronic hepatitis (n = 17)	
Age (yrs)	58.9 ± 10.4
Gender (M/F)	8/9
Etiology of liver disease	
HBV/HCV/NBNC	0/17/0
Healthy controls (n = 41)	
Age (yrs)	46.1 ± 19.1
Gender (M/F)	16/25

Note: Results except for AFP and DCP are expressed as means ± SD. AFP and DCP values are expressed as range (median). The reference range of normal values for the laboratory values: AFP < 10 ng/mL, DCP < 40 mAU/mL. M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-B non-C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

2.2. Isolation of PBMC and $CD4^+$ T cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma–Aldrich, St. Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. $CD4^+$ T cells were isolated from fleshly isolated PBMC by negative magnetic selection using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated $CD4^+$ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE, anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpha Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells " Plasmacytoid Subset " and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset " (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and intracellular staining

To determine the frequency of $CD4^+CD25^{high}$ T cells and the surface marker profile, $CD4^+$ T cells (at least 2×10^5 cells/tube) were stained with mAbs in the above described panel for 30 min on ice. Apropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2×10^5 $CD4^+$ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37 °C in a humidified CO_2 incubator for 4 h. The activated cells were first incubated with anti-CD4-PerCP for 15 min on ice, followed by fixation and permeabilization of the activated cells for 20 min at room temperature with BD Cytofix/Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD

Biosciences) for 15 min at room temperature. Apropriate isotype controls were included for each sample.

2.5. Flow cytometric analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6. Statistical analysis

Data are indicated as means ± SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric U test. $P < 0.05$ was considered significant.

3. Results

3.1. Frequencies of $CD4^+CD25^{high}$ T cells

To evaluate the frequencies of $CD4^+CD25^{high}$ T cell subsets that contain Tregs, MACS-sorted $CD4^+$ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and B). Although the frequencies of $CD4^+CD25^{high}$ T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that $CD4^+CD25^{high}$ T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and cytokine production of the $CD4^+CD25^{high}$ T cell subset in HCC patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22–24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was larger than that of $CD4^+CD25^{low/negative}$ subset, and it was also significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients (Fig. 2B). Thus, not only is the number of $CD4^+CD25^{high}$ T cells in HCC patients larger, but also the frequency of $Foxp3^+$ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of $CD4^+CD25^{high}$-$Foxp3^+$ T cell subset was quantitated following the stimulation with PMA/ionomycin using the specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the $CD4^+CD25^{high}$ subsets. In addition, the levels of IL-4 and IL-10 were high in the $CD4^+CD25^{high}Foxp3^+$ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the $CD4^+CD25^{high}Foxp3^+$ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.3. Phenotypes of the $CD4^+CD25^{high}$ T cell subset in HCC patients

To determine the phenotypical properties of $CD4^+CD25^{high}$ T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RA, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ cells were higher in the $CD4^+CD25^{high}$ T cell subset in all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$) (Fig. 3A and B). The percentage of $CD45RO^+$ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the $CD4^+CD25^{high}$ T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

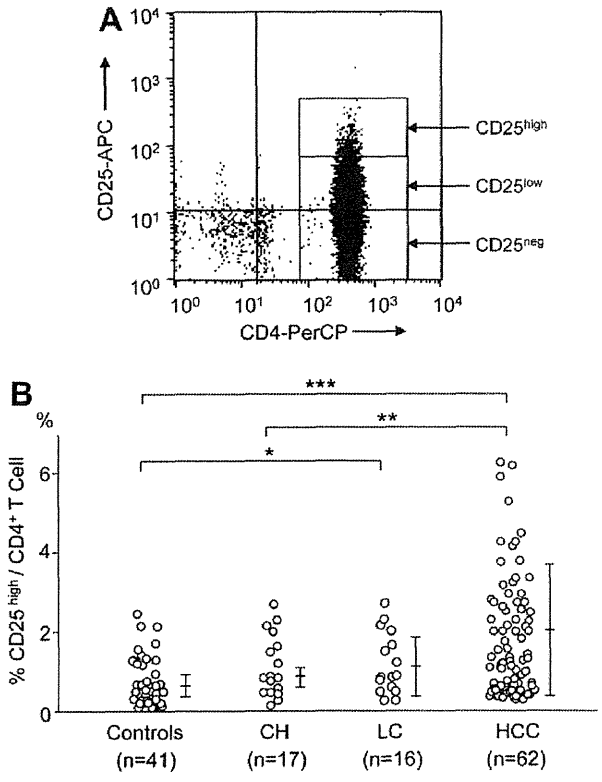

Fig. 1. Frequencies of $CD4^+CD25^{high}$ T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of $CD4^+CD25^{high}$ T cells in the peripheral blood of HCC ($n = 62$), LC ($n = 16$), CH ($n = 17$) patient, and healthy controls ($n = 41$). Percentages for individual patient analyzed are shown. The percentages represent the proportions of $CD4^+CD25^{high}$ T cells in total $CD4^+$ cells. The prevalence of $CD4^+CD25^{high}$ T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

3.4. $CD4^+CD25^{high}$ T Cell subset and dendritic cells of HCC patients

Several reports have suggested that the $CD4^+CD25^{high}$ T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD123-PC5 Dendritic Cells "Plasmacytoid Subset" and IOTest Conjugated Antibodies – (CD14 + CD16)-FITC/CD85k(ILT3)-PE/CD33-PC5 Dendritic Cells "Myeloid Subset". HCC patients were divided into two groups according to the frequencies of $CD45RO^{positive}$ cells in $CD4^+CD25^{high}$ T cell subsets ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained >83.8% positive cells in $CD4^+CD25^{high}$ T cells. The frequencies of $CD123^+$ plasmacytoid DCs were significantly higher in $CD45RO^{++}$, group ($P < 0.05$) (Fig. 5A and B), although those of $CD33^+$ myeloid DCs were not correlated with the subsets in $CD4^+CD25^{high}$ cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of $CD4^+CD25^{high}$ T cells.

3.5. $CD4^+CD25^{high}$ T cell subset and tumor progression

To evaluate the association between $CD4^+CD25^{high}$ T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child-Pugh scores

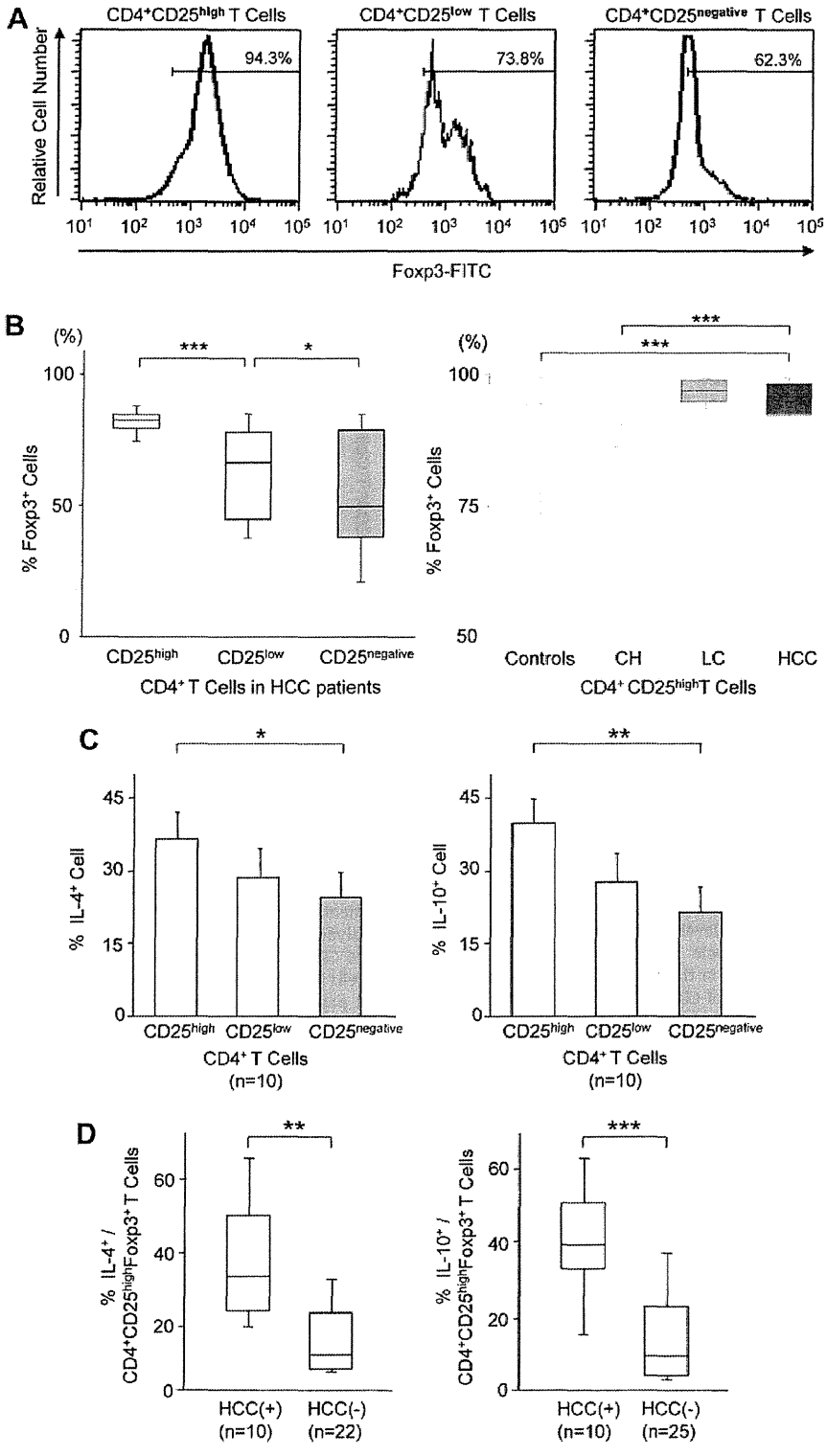

Fig. 2. Analysis of intracellular Foxp3 expression and cytokine production in $CD4^+$ $CD25^{high/low/negative}$ T cell subsets in HCC patients. (A) Representative expression of Foxp3 in $CD4^+$ T cells from an individual subject. Intracellular Foxp3 was stained following membrane permeabilization. Intracellular Foxp3 was detected by the specific mAb. (B) Statistical analysis in the left side panel shows that the percent of $Foxp3^+$ cells in the $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{low/negative}$ T cell subsets, and in the right side panel shows that that of $CD4^+CD25^{high}$ T cell subset in HCC patients was significantly larger than that of $CD4^+CD25^{high}$ T cells in healthy controls and CH patients. (C) Statistical analysis shows that the levels of Th2 cytokines IL-4 and IL-10 were remarkably high in the $CD4^+CD25^{high}$ T cell subset. (D) Comparison of intracellular cytokine production in $CD4^+CD25^{high}$ T cell subsets between patients with and without HCC. Healthy controls, patients with chronic hepatitis and liver cirrhosis were included in the HCC (−) column. IL-4 and IL-10 levels were higher in the $CD4^+CD25^{high}$ T cell subset in HCC patients. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

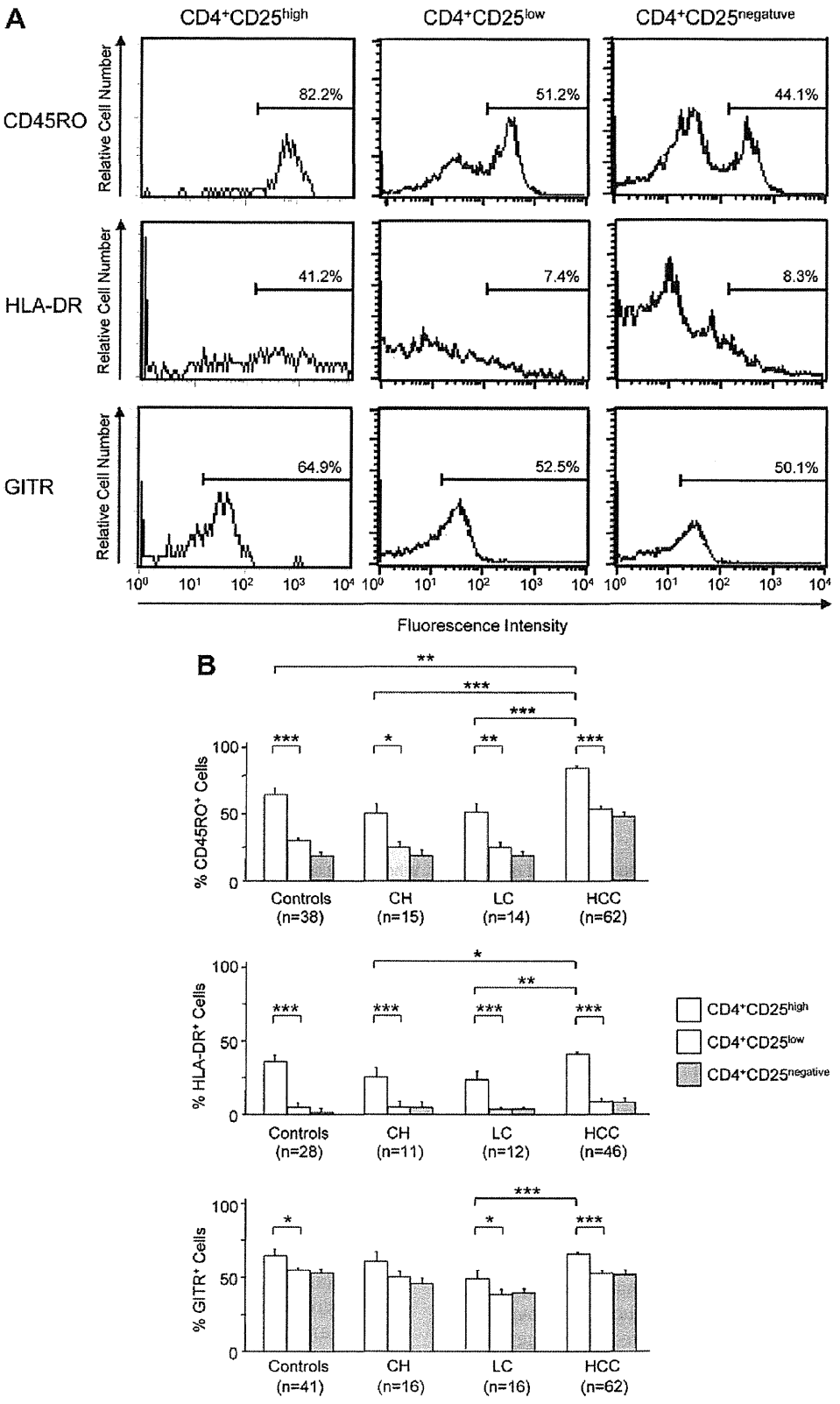

Fig. 3. Phenotypic analysis of $CD4^+CD25^{high/low/negative}$ T cell subsets in HCC patients. Fleshly isolated $CD4^+$ T cells (at least 2×10^5 cells/tube) from HCC patients were labeled with anti-CD4, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR7, anti-CTLA-4, anti-HLA-DR and anti-GITR mAbs. (A) Representative CD45RO, HLA-DR, and GITR expression profiles in $CD4^+$ T cell subsets that differ in CD25 expression. (B) Statistical analysis shows that the proportions of $CD45RO^+$, $HLA\text{-}DR^+$ and $GITR^+$ were elevated in the $CD4^+CD25^{high}$ T cell subsets of all patient groups compared to the $CD4^+CD25^{low/negative}$ T cell subsets, except for $GITR^+$ cells in CH patients ($P < 0.05$). The percentage of $CD45RO^+$ cells in HCC patients was elevated compared to the patients with advanced liver diseases and healthy controls. *Indicates $P < 0.05$, **indicates $P < 0.01$ and ***indicates $P < 0.001$.

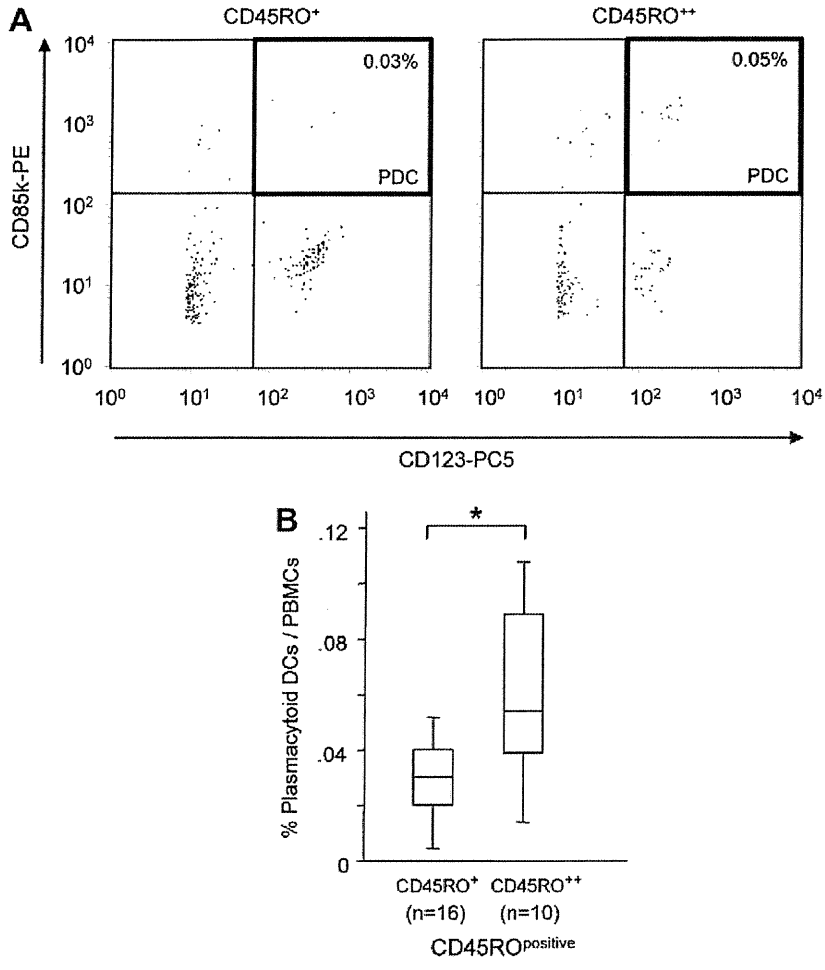

Fig. 4. Frequencies of plasmacytoid DCs in peripheral blood of HCC patients. Whole blood cells were analyzed by flow cytometry following staining with a combination of the mAbs. HCC patients were divided into two groups according to the frequencies of $CD45RO^{positivee}$ cells in $CD4^+CD25^{high}$ T cell subset ($CD45RO^+$ vs. $CD45RO^{++}$). Patients with $CD45RO^{++}$ contained > 83.8% positive cells in $CD4^+CD25^{high}$ T cells. (A) Representative dot plots of plasmacytoid DCs. Plasmacytoid DCs of $CD45RO^+$ group are shown in the left panel and $CD45RO^{++}$ group in the right panel. (B) Statistical analysis shows that the frequencies of plasmacytoid DCs were significantly higher in $CD45RO^{++}$ group. *Indicates $P < 0.05$.

and fibrosis stages between two groups as described above. The levels of serum AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ group (Fig. 4). Others were not significantly different between two groups. These results imply that a subset of Tregs may contribute to the progression of liver tumors.

4. Discussion

$CD4^+CD25^{high}$ $Foxp3^+$ regulatory T cells have been shown to increase in patients with malignancies to suppress the immune responses. In this study, we provide evidence that patients with HCC have increased frequencies of $CD4^+CD25^{high}$ T cells in their peripheral blood compared to healthy controls and chronic hepatitis patients. A large proportion of $CD4^+CD25^{high}$ T cells expressed Foxp3 and produced Th2 cytokines. We also showed that $CD4^+CD25^{high}$ T cells expressed high levels of CD45RO, HLA-DR and GITR, and, interestingly, the T cell frequencies expressing these surface molecules were associated with plasmacytoid DC numbers and maximum tumor diameters in HCC patients.

There are several reports of elevated numbers of Treg cells in the peripheral blood and tumor tissues of patients with different types of cancer [3–12]. The study of Unitt et al. provided the first report of increased $CD4^+CD25^+$ T cell frequency within tumor tissue compared to non-tumor tissue in HCC patients [13]. Ormandy et al. showed that the frequency of $CD4^+CD25^{high}$ T cells in peripheral blood of patients with HCC was significantly higher (3.92 ± 3.3%) than in healthy donors (1.17 ± 0.87%) and liver cirrhosis patients (0.78 ± 0.43%) [3]. Our data revealed that a minimal increase in $CD4^+CD25^{high}$ T cells was detected in LC patients and more pronounced changes were found in HCC patients.

We showed that higher percentages of $CD4^+CD25^{high}$ T cells produced Th2 cytokines IL-4 and IL-10 in HCC patients. Tregs were recently observed to produce IL-10 [25–27], which can be a major mediator of immune suppression [28–30]. Voo et al. reported that Tregs in the peripheral blood of healthy donors secreted IL-10 but not IL-2, IFN-γ, or IL-4 [31]. Schmitz-Winnenthal et al. demon-

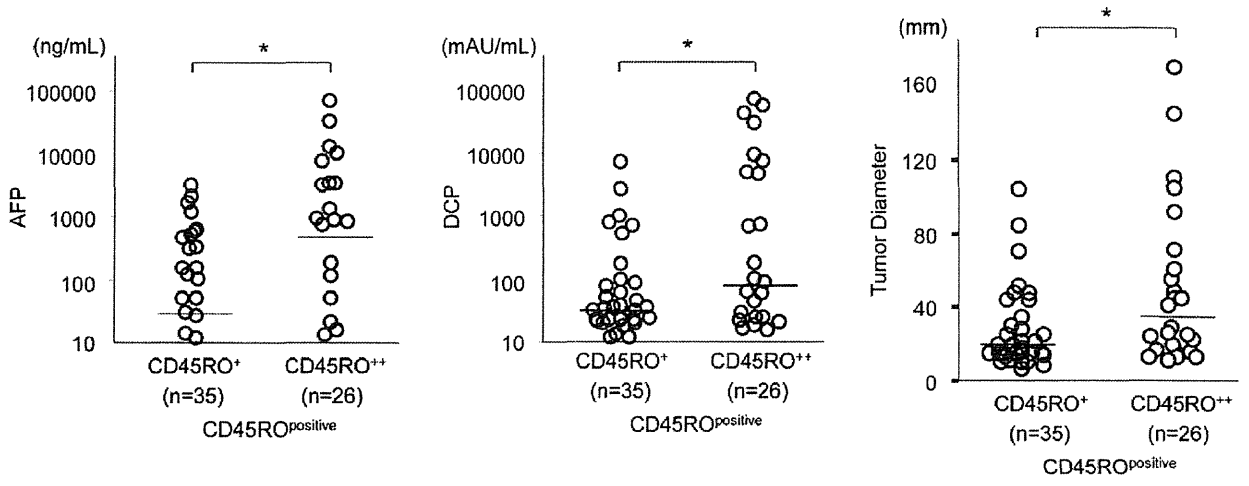

Fig. 5. Prevalence of $CD4^+CD25^{high/low/negative}$ T cell subsets and tumor progression. The levels of AFP and DCP and the maximum tumor diameters in $CD45RO^{++}$ group were larger than those in $CD45RO^+$ groups. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyl prothrombin. *Indicates $P < 0.05$.

strated the presence of Treg secreting IL-10 but not IL-4 or IFN-γ upon antigen recognition in chronic pancreatitis patients [32]. The present data demonstrated that larger numbers of Tregs produced not only IL-10 but also IL-4 in HCC patients, which may contribute to the strong immunosuppressive properties of the T cells in liver malignancies.

It appears that Tregs consists of heterogenous populations within $CD4^+$ T cells, and that a subset of $CD4^+CD25^{high}$ T cells could be subdivided into different functional subsets based on the expression of various surface molecules [6]. The proportions of Tregs expressing these molecules are reported to be different in the various forms of cancer. The prevalence of $CD45RO^+$ and $GITR^+$ Treg cells is higher in $CD4^+CD25^{high}$ T cells than in $CD4^+CD25^{low/negative}$ T cells in renal cell carcinoma [4]. In head and neck squamous cell carcinoma, however, $CD4^+CD25^{high}$ T cells express CTLA-4, Foxp3, and CD62L but little GITR, and $CD25^{low/negative}$ T cells express intermediate to high levels of GITR and HLA-DR [8]. Our study showed that Tregs in HCC patients expressed significantly higher levels of CD45RO, HLA-DR and GITR compared to $CD4^+CD25^{low/negative}$ cells, suggesting that the activated populations of Tregs may contribute to the establishment of immunosuppressive microenvironments.

Little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated numbers of Treg cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells [20,33]. In peripheral blood, there are two distinct populations of DCs which can be distinguished based on phenotypical and morphological characteristics; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18,34]. Our data demonstrated that higher frequencies of $CD45RO^+CD4^+CD25^{high}$ T cells were associated with higher frequencies of pDCs in the peripheral blood of HCC patients. When the tumor antigens are assumed by pDCs through Toll-like receptor 9 (TLR9) via receptor-mediated endocytosis, secretions of pro-inflammatory cytokines, such as type I interferons (IFNs), would be caused. On the contrary, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape. They exhibit reduced IFN-α production upon TLR9 stimulation and can induce IL-10 producing $CD4^+$ and $CD8^+$ Treg [35,36]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the tumor and by limiting anti-tumor cytolytic activity through induction of $CD8^+$ Treg.

Concerning the association of Tregs and prognosis, it has been reported that an increased number of circulating Tregs predicts poor survival of patients with renal cell carcinoma [4], gastric and esophageal cancers [7], myelodysplastic syndrome [37] and HCC [11]. In addition, tumor-infiltrating Tregs were associated with reduced survival in ovarian cancer [12] and HCC patients [1]. In addition, we found that $CD45RO^+CD4^+CD25^{high}$ T cell subset was associated with larger tumor burdens, implying that a subset of Tregs may contribute to the promotion of tumor cell growth in the liver. However, it is also well possible that this just reflects stronger activation caused by a larger amount of antigen.

We performed the functional evaluation of Tregs derived from HCC patients by incubating with responder $CD4^+CD25^-$ T cells (Tresp). We observed that $CD45RO^+$ $CD4^+CD25^{high}$ T cells of HCC patients did not suppress the proliferation of responder T cells when co-cultured at Treg/Tresp ratios of 1:2 and 1:8 (data not shown). In contrast, Hoffmann et al. confirmed that the $CD45RA^+$ $CD4^+CD25^{high}$ T cells of healthy volunteers give rise to a homogeneous and highly suppressive Treg cell population, whereas $CD45RA\text{-}CD4^+CD25^{high}$ T cells generate cell lines with mixed phenotype and function [38]. Although the reasons of these conflicting data were not clarified in the current study, cell viability, apoptosis susceptibility, involvement of Th1 cytokines, and interaction to helper T cell subsets of Tregs obtained from HCC patients need to be evaluated in the future experiments.

This study may be helpful for a better characterization of Treg subsets in the peripheral circulation of patients with HCC, which may establish the immunosuppressive environment to promote tumor progression. Furthermore, to gain insights into changes in the Treg subsets

during the therapeutic option may lead to more effective immunotherapies against cancer and may improve prognosis.

Conflict of interest

None declared.

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全国集計からみた原発性胆汁性肝硬変の疫学的動向

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索引用語：原発性胆汁性肝硬変，疫学，予後，オーバーラップ症候群，肝細胞癌

1 はじめに

本邦における原発性胆汁性肝硬変(primary biliary cirrhosis, PBC)の全国調査は厚生労働科学研究補助金，難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班(現研究代表者，坪内博仁) PBC分科会(現分科会会長，中沼安二)により1980年から継続的に実施されてきた．

本稿では，第1回(1980年実施)から第14回(2009年実施)全国調査まで30年におよぶ全登録症例7,376例の集積データをもとに，本邦におけるPBCの疫学的動向について概説し，PBC/自己免疫性肝炎(autoimmune hepatitis, AIH)オーバーラップ症例と悪性腫瘍の発生についても言及する．

2 本邦における推定PBC患者数と諸外国の状況

1989年より症候性PBCは厚生労働省特定疾患として公費負担が認められた．2008年度特定疾患医療受給者証交付件数による医療受給者数は16,112人であり[1]，無症候性PBCを含めると本邦における年間推定発生数は約500人，推定患者数は約50,000～60,000人(2008年度)となる．

諸外国の報告では，罹患率(発生数)は0.7～49人／人口100万人，有病率(患者数)は6.7～402人／人口100万人とされているが，国および地域により大きな相違がある[2,3]．イギリス，スウェーデン，北米など北半球では罹患率および有病率ともに高率である一方，オーストラリアでは低率でありアフリカの一部やインドにはほとんどみられない．また同じ国内においても地域差がある．これらの違

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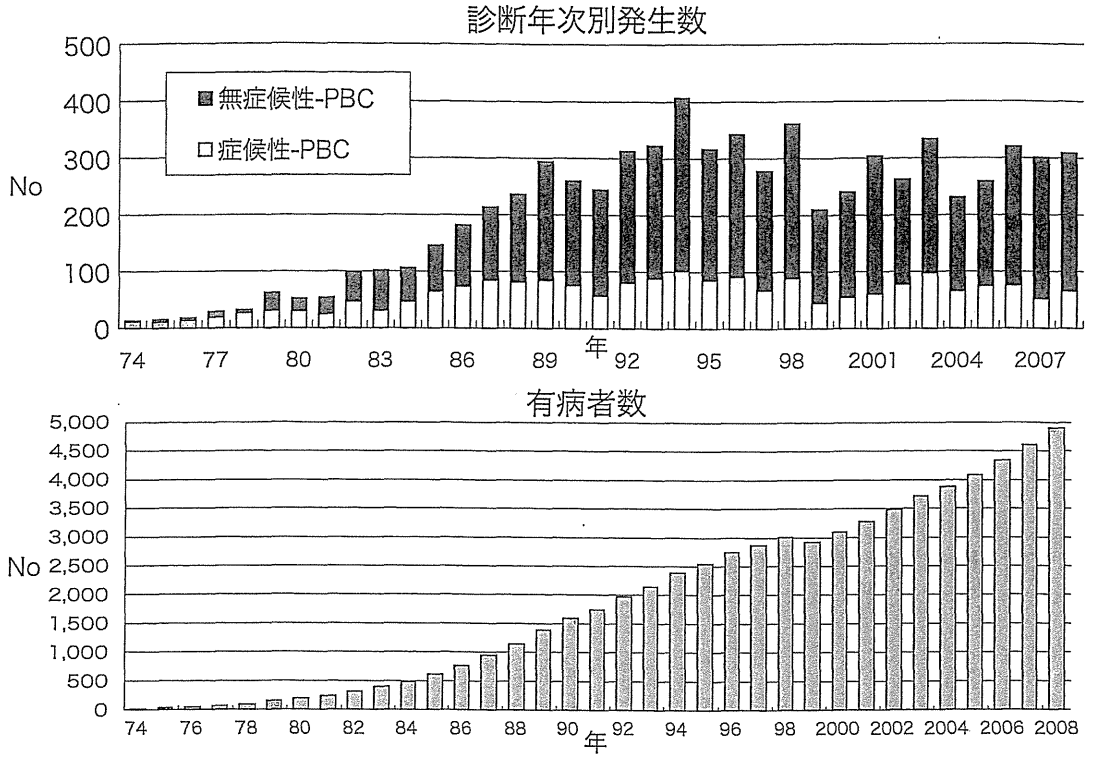
診断年次別発生数
500
400
300
200
100
0
No
無症候性-PBC
症候性-PBC
74 77 80 83 86 89 92 95 98 2001 2004 2007
年
有病者数
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
No
74 76 78 80 82 84 86 88 90 92 94 96 98 2000 2002 2004 2006 2008
年

図1

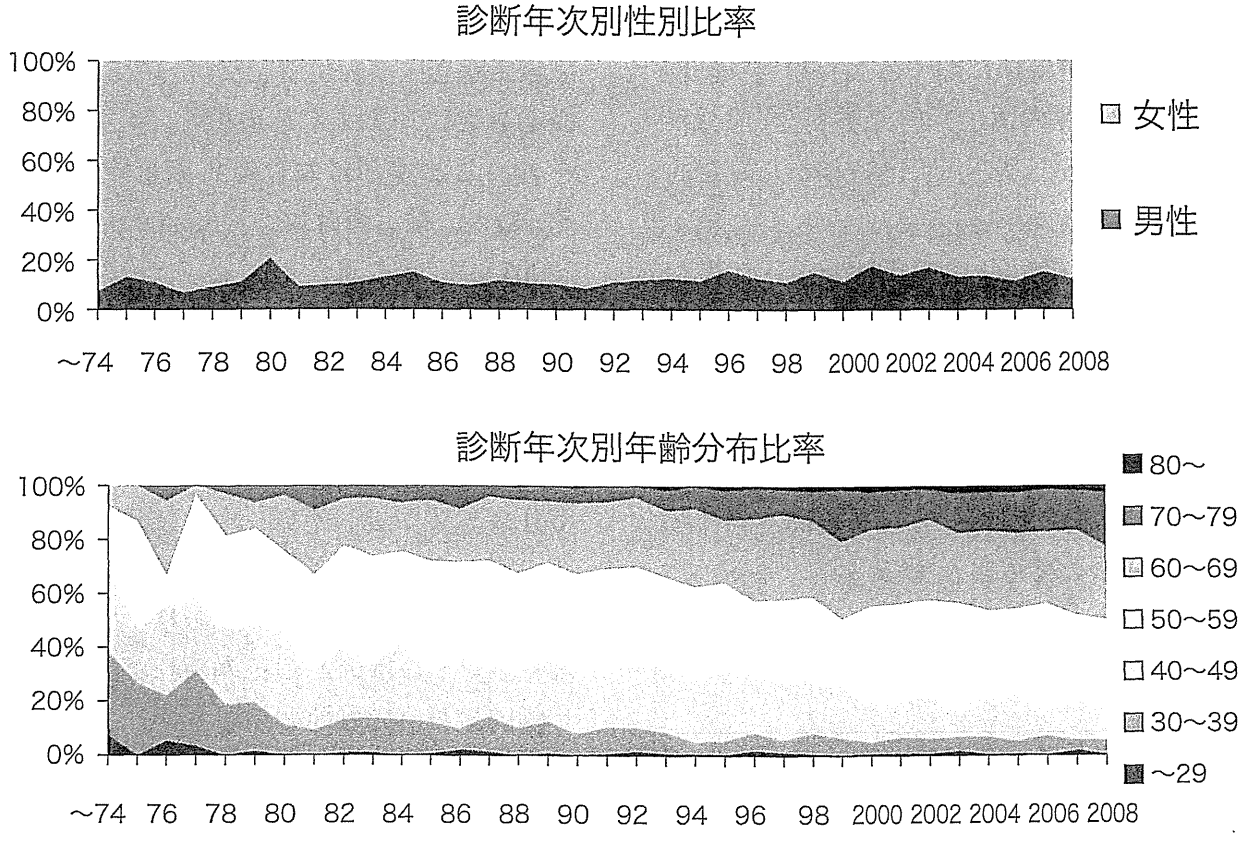
診断年次別性別比率
100%
80%
60%
40%
20%
0%
女性
男性
~74 76 78 80 82 84 86 88 90 92 94 96 98 2000 2002 2004 2006 2008
診断年次別年齢分布比率
100%
80%
60%
40%
20%
0%
80~
70~79
60~69
50~59
40~49
30~39
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図2

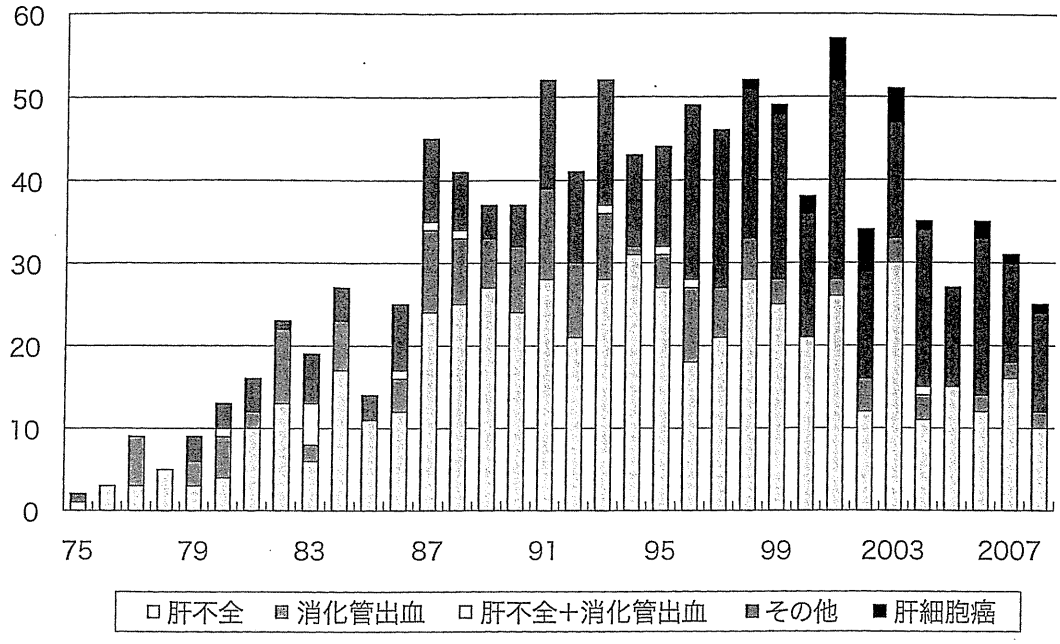

図3　死亡年次別にみた死亡数と死因

いは医療側の診断能力の問題または遺伝学的因子，感染因子，生体外異物による環境因子などPBCの病因因子が関与している可能性もあるが明らかではない．

3　全国調査からみた疫学的動向

全国調査のデータは，全国の専門施設を対象として登録症例の追跡予後調査を継続的に実施して得られた統計資料であり，本邦全PBC症例を把握しているわけではないものの本邦における疫学的動向を把握するために有用とされている．診断年次別発生数は1980年の調査開始以来増加傾向にあったが，1990年代以降は横ばいで推移している．新たに診断される症例のうち約80％以上は無症候性PBCである(図1)[4]．PBCの疾患概念および診断基準が確立し本症に関する知識が医療者に浸透するにしたがい，また診断根拠の一つである抗ミトコンドリア抗体測定が簡便に行えるようになった時期に相応してPBCと診断される症例は漸増している．近年は検診時や他疾患受療時に肝機能検査値異常を契機として診断される無症候性PBC例が増加している．PBCの有病者数は，年々増加しているが(図1)[4]，無症候性PBC例の増加および無症候性，症候性PBCともに予後が改善していることが，有病者数の増加に影響しているものと考えられる．

男女比は1：7で女性に多く，診断時平均年齢は56歳(男性59歳，女性55歳)であり，男女比に大きな変化は認められないが，診断時年齢は年々高齢化している(図2)[4]．

4　予後の変遷

原疾患における主な死因は肝不全と消化管出血でかわりはないが，1990年代以降，原疾患以外の死因による死亡数が増加し2000年代になると肝不全，消化管出血による死亡数は減少傾向となっている．長期生存例の増加により少数例ではあるが，肝細胞癌を合併し癌死に至る症例もある(図3)[5]．

予後は診断時臨床病期により異なり無症候性PBCの5年生存率は98％，10年生存率は93％で，症候性PBCではおのおの80％，

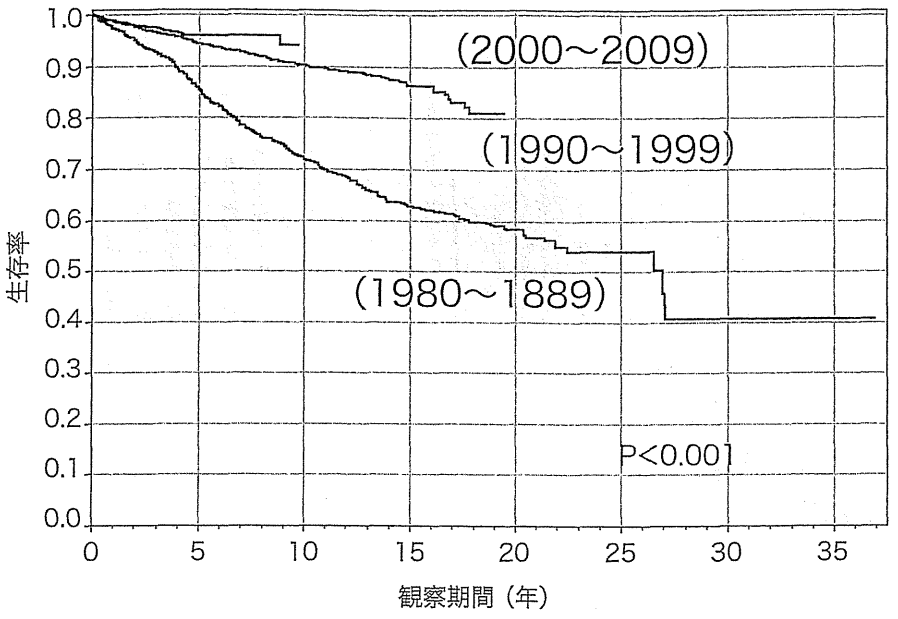

図4　年代群別生存率

66％である．無症候性PBCには病期が進行しない予後良好な経過をたどる群と徐々に進行する群が存在する．診断時無症候性であった症例の約80％は無症状のまま経過し，そのうちの99％は15年以上生存し予後良好な経過を示す．一方，無症候性PBCの約20％は症候性に移行しうち20％が死亡する[5]．

30年におよぶPBC全国調査登録症例を年代別に解析したところ予後は明らかに改善傾向にある．5年生存率では1989年までに診断された群85.4％，1990年から1999年に診断された群94.2％，2000年以降に診断された群95.8％と年代を経るごとに有意に高率であった(図4)[4]．診断時の臨床病期別にみても無症候性PBC，症候性PBCとも年代別にみて有意に予後は改善していた．予後良好な群の占める割合が無症候性PBCの大部分を占めるようになったこと，ウルソデオキシコール酸をはじめとする内科的治療および一般的な肝不全に対する治療また消化管出血に対する内視鏡的治療などの相乗的効果により，各臨床病期における予後が改善しているものと推測される．しかしながら，診断時すでに総ビリルビン値5 mg/dl以上の発黄例，またScheuer分類Ⅲ，Ⅳ期の組織学的進行例では年代的にみても有意な予後改善は認められておらず，これらの群では現行の内科的治療効果が乏しいことが推察される[4]．

5　PBC/自己免疫性肝炎（autoimmune hepatitis, AIH）オーバーラップ症候群

PBCには非定型例のあることが知られており診断および治療選択に苦慮する場合がある．肝炎の病態を併せ持ちALTが高値を呈する病態には副腎皮質ステロイドの投与によりALTの改善が期待できるため，PBCの典型例とは区別しPBC/自己免疫性肝炎(autoimmune hepatitis, AIH)オーバーラップ症候群と呼称される．

PBC全国調査登録例はすべて本邦PBCの診断基準に合致した症例であるが，登録例中にはこれらPBC/AIHオーバーラップ症候群が混在している可能性がある．第6回～11

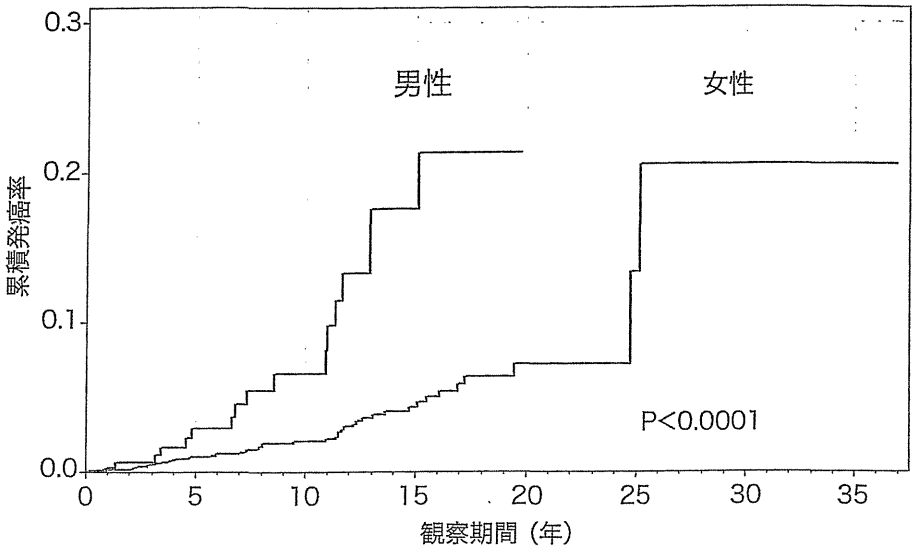

図5　男女別肝細胞癌累積発癌率

回調査までの登録症例を対象として解析した結果[6]では，登録症例全体の72％を占める群(AMA陽性または抗PDH抗体陽性でANA陽性またはSMA陽性)のなかでγglobまたはIgGが2.0 g/dl以上かつALTが正常上限の2倍以上を満たす症例は約13％あり，全体の7％を占める群(AMA陰性かつ抗PDH抗体陰性でANA陽性またはSMA陽性)中には同条件を満たす症例は約16％存在しており，登録症例全体として約10％にPBC/AIHオーバーラップ症候群を疑う症例が含まれていることが推定された．

6　悪性腫瘍の合併

第14回全国調査において，PBCの診断時に合併する悪性腫瘍は3.1％であった．その内訳は肝臓24％，胃17％，大腸11％，乳腺10％，子宮5％，甲状腺5％，血液疾患5％，卵巣3％，肺2％，副腎1％，その他17％となっている[5]．

従来PBCにおける肝細胞癌の発生は比較的稀とされてきたが，長期生存例が漸増している中，経過中に肝細胞癌発症した症例が集積されつつある．第1回～第14回全国調査登録症例のうち，経過中に肝細胞癌の発生を確認した53例(肝炎ウイルスマーカー陽性例を除外)について検討したところ，累積発癌率はPBC診断後10年で男性6.5％，女性2.0％と明らかな性差が認められ(図5)，発癌症例は予後不良であった．発癌の背景因子について検討したところ，女性例では組織学的病期進展が発癌の危険因子であったが男性では他の要因の関与が示唆された．Shibuyaら[7]は多変量解析を用いた報告で高齢，男性，輸血歴を発癌の危険因子として，また別の報告では組織学的進展度を危険因子としてあげており[8]，これらに相当するPBCにおいては定期的に肝細胞癌のスクリーニング検査を行う必要性があると考えられる．

7　おわりに

原発性胆汁性肝硬変(PBC)全国調査の30年に及ぶ長期追跡症例の検討から本邦におけるPBCの疫学的動向について概説した．おおむねPBCの予後は改善しているが，病期進展例では最近の治療を駆使しても有意な予

後改善は得られず，これらの群では肝移植に頼らざるを得ない状況にあり新しい観点からの治療法の開発が待たれるところである．

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