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 ($P < 0.01$).

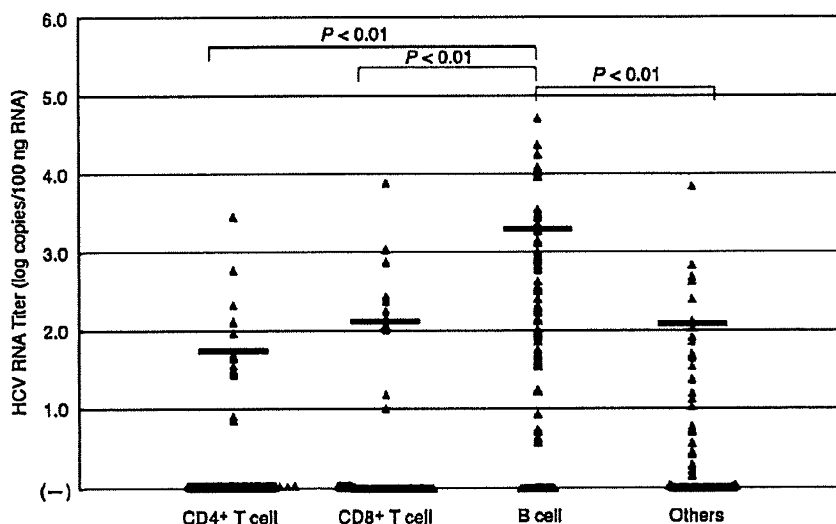

Fig. 1. HCV RNA titers in various compartments of lymphoid cells. Bold bars indicate mean values.

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

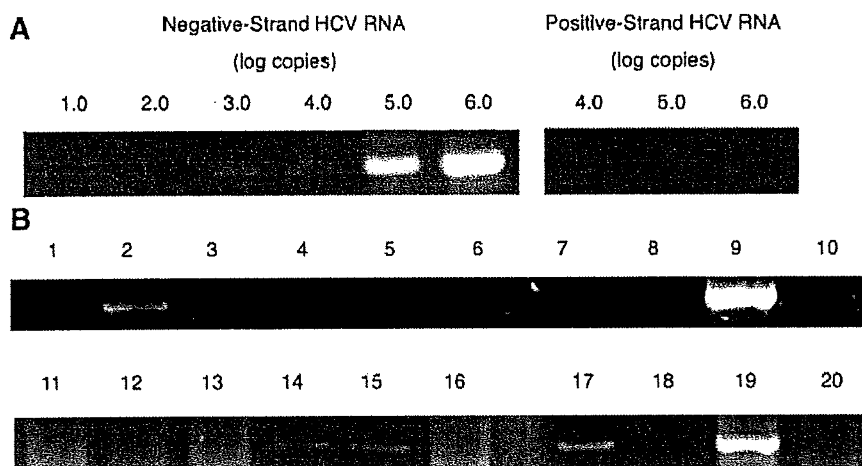

Fig. 2. A: Ethidium bromide staining of products of strand-specific RT-PCR for negative- and positive-strand HCV RNA. B: Strand specific RT-PCR for negative-strand HCV RNA from B cells of 16 patients. Positive control for negative-strand HCV RNA (lanes 9 and 19) and negative control for positive-strands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

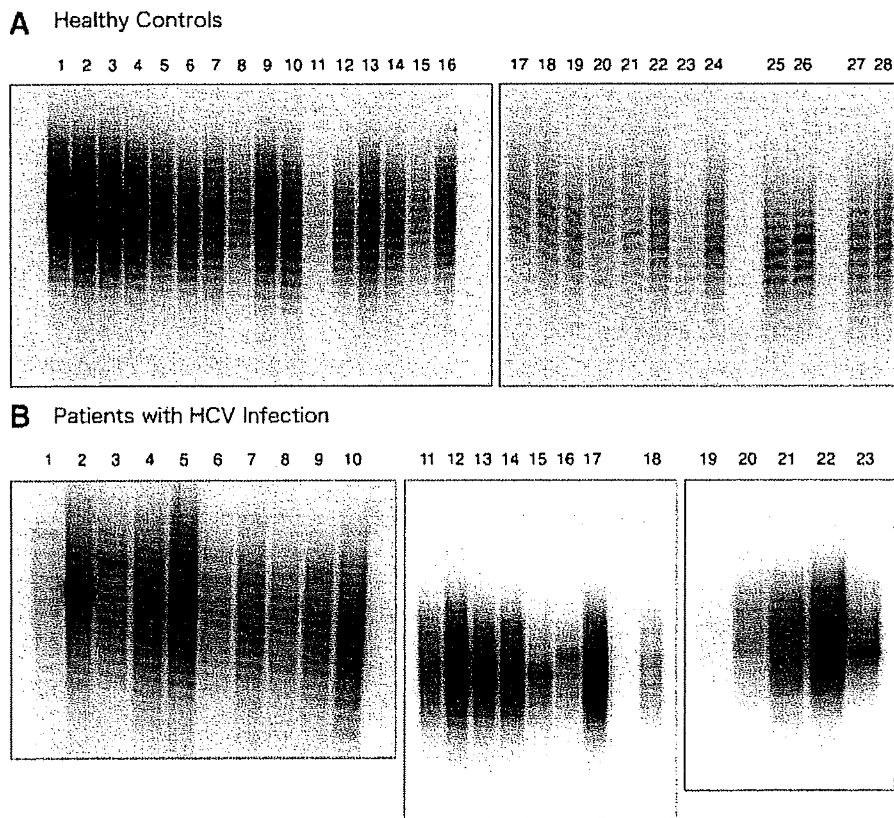

Fig. 3. Ig fingerprinting assay of (A) PBMCs from 28 healthy subjects and (B) B cells from 23 patients infected with HCV.

(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 LCFQAVHL-I CDAFCIIS*D -ESA-HRRGV FCCPTISFAS NETY--Q--- WS-AD--H-- IT--EP--CT -ESQ
C10  -------P-- ---I--RPEP VNPTPDFCTL IEG-ALSREA AHL*IQ-VPA 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 -ESC
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 PIHSSPFFGA AGSSSSSNY* NSHQGQC-RG T-SPK
```

(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-X-FLNSTV ATT*C----- ---R---A-- ----VCRC-I G----A-L-- -T---TTVSA ---H--*P-- RG---SADTT AG--VT---- ----
HC7  ----Q-S--- VPYLQAANSR TVIHSRVLSS QAVHL-IQRV LGIVSGDGES ALHGVCVVFI TSII*YNCHP LQPLAWSLAD PVH-IASEGE SRGCTGESCG 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 RDNSFKHMFS 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-XGGVI--I -IVPTPKIFH T-IYGRVLGS QAVHLQIQRV LGI-PGDGES ALHG-CIVFI TSIIPYNCHP LQPLAWSLAD PVHAIATEGE SRRC
HC25 ----Q-S--- V-XVPRI-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 PKVDPDAACD 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 SRGCTGESCG PPRL
HC21     X X PQ     X  VVFP HTIVEI*FSH SNRQPLSSG  RHVHLHI AC YWSRLXXW*I GPSRSLHSSS YPHLLI*LPP  PAPCLEPGG HSACQNX*SV NAMR
HC2  ----Q-S--- VPQRNCSHLY FS-TVTHCRV LGSQAVHL-I Q*VLGVVSGD -ESALHRVHI VFLTSILLYV GFPLQPLPWP ADPAH-IATE GESRGCTGES QGPD
HC23 ----Q-S--- V-XMCSSYHH 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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CLINICAL STUDIES

Identification and characterization of IgG4-associated autoimmune hepatitis

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Keywords
autoimmune hepatitis – IgG4 – steroid treatment

Abbreviations
AIH, autoimmune hepatitis; AIP, autoimmune pancreatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus; HPF, high-power field; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; Th1, T helper type 1; Th2, T helper type 2.

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Abstract

Background: Autoimmune hepatitis (AIH) and autoimmune pancreatitis (AIP) share clinical and pathological features such as high serum levels of immunoglobulin (Ig) G and autoantibodies, and lymphoplasmacytic infiltration, suggesting the presence of common immunological abnormalities. However, little is known about the possible involvement of IgG4, a hallmark of AIP, in AIH. *Aims:* In this study, we examined whether the IgG4 response contributes to the histopathological and clinical findings in AIH. *Methods:* Liver sections from 26 patients with AIH, 10 patients with primary biliary cirrhosis (PBC), three patients with primary sclerosing cholangitis (PSC) and 20 chronic hepatitis patients with hepatitis C virus (HCV) infection were immunostained for IgG4. We investigated the relationship among the histopathology, the responses to steroid therapy and the IgG4 staining. *Results:* Nine of the 26 liver specimens from patients with AIH showed positive staining for IgG4 whereas none of the 10 samples from patients with PBC, the three samples from patients with PSC or the 20 samples from patients with HCV hepatitis were positive. Patients with IgG4-positive AIH also showed increased serum levels of IgG. The numbers of T cells, B cells and plasma cells were significantly increased in the livers of patients with IgG4-positive AIH as compared with those patients with IgG4-negative AIH. Patients with IgG4-positive AIH also showed a marked response to prednisolone therapy. *Conclusions:* AIH may be classified into either an IgG4-associated type or an IgG4 non-associated type with the former showing a marked response to prednisolone treatment.

Autoimmune hepatitis (AIH) is an organ-specific disease of the liver that is characterized by hypergammaglobulinaemia, autoantibodies in the serum and by the presence of interface hepatitis and plasma cell infiltration on histological examination (1). The pathogenic mechanisms accounting for the development of AIH are still unknown. Because AIH is associated with certain human leukocyte antigen subtypes (2) and with the presence of various autoantibodies (2, 3), it is plausible to assume that adaptive immune responses mediated by T cells and B cells are involved. This notion is supported by research showing that antibodies to autoantigens such as soluble liver antigen/liver–pancreas antigen and cytochrome P-450 2D6 are involved in the progression of the disease (3). In addition, recent findings suggest that T-cell-mediated immune responses play a major role in the development of AIH. For example, a predominant infiltration of $CD4^+$ and $CD8^+$ T cells is seen in the livers of patients with AIH (4). These liver-infiltrating T cells produce both T-helper type 1 (Th1) and type 2 (Th2) cytokines that mediate liver damage (5). On the other hand, the number of $CD4^+CD25^+$ regulatory T cells that function to suppress the effector Th1 and Th2 responses is decreased in peripheral blood samples taken from patients with AIH (6). Thus, the pathogenic mechanisms of AIH may partially be explained by both an enhanced effector T-helper response and an impaired regulatory T-cell response. In contrast to the role played by T cells, our knowledge regarding the role of B cells and plasma cells infiltrating the AIH lesions is very limited.

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Recent studies of the immunopathogenesis of autoimmune pancreatitis (AIP) have shown results that may potentially be applicable to AIH (7). For example, as in the case of AIH, patients with AIP show elevated levels of serum immunoglobulin (Ig) G and autoantibodies (8). Lymphoplasmacytic infiltration of T cells and plasma cells is seen in the pancreas of patients with AIP, findings that are similar to those of the liver of patients with AIH (9). Furthermore, both AIP and AIH respond well to steroid therapy (1, 10). Thus, AIH and AIP appear to share clinical and histological features. It is now generally accepted that patients with AIP have elevated levels of serum IgG4 (11) and that plasma cells expressing IgG4 are abundantly seen not only in the pancreas but also in the other involved organs (12). More importantly, the presence of IgG4-expressing plasma cells in the liver has now been convincingly linked to cholangitis and hepatitis (13–15). In this respect, IgG4 itself does not seem to be responsible for the development of liver damage because this IgG subtype does not cause cell-mediated lysis owing to poor binding activity to complement (16). It is possible, however, that abnormal immunological environments leading to enhanced IgG4 responses, rather than IgG4 itself, underlie the pathogenesis of the liver damage seen in AIH. Given the similar clinical and pathological features between AIH and AIP, we asked the question as to whether these two autoimmune disorders share a common pathophysiology. To address this, we examined the IgG4 expression in the livers of patients with AIH. Our results identify a subtype of AIH that is characterized by the infiltration of IgG4-expressing plasma cells and by a marked response to steroid therapy.

Methods

Patients

Twenty-six AIH patients who met the international criteria for the diagnosis of AIH (17) were enrolled in this study from October 2002 to May 2007. All patients were admitted to Kinki University Hospital or two affiliated hospitals. Ten primary biliary cirrhosis (PBC) patients, three primary sclerosing cholangitis (PSC) patients and 20 chronic hepatitis patients with hepatitis C virus (HCV) infection were also studied. The diagnosis of PBC was made based on established criteria [i.e. at least three of the following: alkaline phosphatase (ALP)-γ-glutamyl transpeptidase (γ-GTP) above the upper limit of normal; antimitochondrial antibodies positive at a titre of 1:20; increased serum levels of IgM; the absence of biliary obstruction as assessed by ultrasonography, computed tomography or cholangiography; or a compatible liver biopsy] (18). The diagnosis of PSC was made based on the findings of endoscopic retrograde cholangiography and liver biopsy (19). The status of hepatitis B virus (HBV) and HCV infection was determined by HB surface antigen and HCV antibody tests. None of the patients with AIH, PBC or PSC were positive for HBV or HCV infection. Ethical permission for this study was granted by the review board of Kinki University.

Histopathology and immunohistochemistry

Liver specimens were obtained percutaneously with an 18 G needle under ultrasound guidance before starting the treatment. The mean length of the specimen was 1.5 cm and each contained six to 15 portal tracts. Liver tissues were fixed in 10% buffered formalin phosphate and embedded in paraffin, after which 4 μm sections were cut and then stained with haematoxylin and eosin, and elastica van Gieson. The sections were evaluated by experienced pathologists blinded to the laboratory and clinical data. Fibrosis was graded as 0 (absent), 1 (periportal fibrosis), 2 (bridging fibrosis), 3 (bridging fibrosis with lobular distortion) or 4 (cirrhosis). For the histological analysis, the following were categorized as either positive or negative (+, −): canalicular cholestasis, portal inflammation, interface hepatitis, ductular proliferation, chronic non-suppurative destructive cholangitis, bile duct loss, rosette formation and collapse of hepatocytes, i.e. dropout of hepatocytes because of massive necrosis. The following were classed as positive or negative depending on the number of events visible per high-power field (HPF): lobular hepatitis (− = 0–3 focal necrosis/HPF; + = > 3 focal necrosis/HPF), plasma cell infiltration (− = 0–9 cells/HPF; + = > 9 cells/HPF); steatosis [(− = 0–30% cells with fatty change/HPF; + = > 30 cells with fatty change/HPF); and eosinophil infiltration (− = 0–4 cells/HPF, + = > 4 cells/HPF)]. Portal inflammation was quantitatively analysed according to the Ishak scoring system (20). Immunostaining for IgG4 was performed in 26 patients with AIH, 10 patients with PBC, three patients with PSC and 20 patients with chronic HCV infection. Liver biopsy specimens from patients with IgG4-associated cholangitis were also stained with anti-IgG4 as a positive control. After deparaffinization and rehydration, all sections on silane-coated slides were pretreated with proteinase K (Dako, Kyoto, Japan) for 20 min. Endogenous peroxidase was blocked in 1% hydrogen peroxide for 3 min using a microwave oven. After a second blocking step with 2% bovine serum albumin, the sections were incubated with a monoclonal antibody to IgG4 (Zymed Laboratories, San Francisco, CA, USA) for 10 min using a microwave oven. Immunostaining for IgG, IgG1, CD3, CD20 and CD38 was performed on samples from the 26 AIH patients as described previously (21, 22). Briefly, sections were incubated with biotinylated antibodies to IgG, IgG1, CD3, CD20 or CD38. Antibodies to IgG, CD3 and CD20 were purchased from Dako, the antibody to CD38 from Novocastra Laboratories Ltd (Newcastle, UK) and the antibody to IgG1 from The Binding Site (Birmingham, UK). An avidin–biotin technique was used for all the immunostaining experiments, with diaminobenzidine tetrahydrochloride used for visualization and haematoxylin for nuclear counterstaining.

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Treatment and follow-up

All AIH patients were initially treated with 30–40 mg/day prednisolone, except for four elderly patients with low-grade activity [as judged by histology and serum alanine aminotransferase (ALT) levels]. None of the patients were treated with azathioprine, 6-mercaptopurine or cyclosporine. Tapering of the prednisolone dose was performed according to an established protocol (1). Serum levels of ALT were monitored every 2 weeks before normalization and every 3 months after normalization. The dose of prednisolone was increased in some patients who showed elevated levels of serum ALT because of the result of a flare-up of their condition. Treatment continued during the observation period and no patients were lost.

Statistical analysis

Fisher's exact test was used to assess the differences in the patient distribution of variables such as gender and concurrent autoimmune diseases. Analysis of variance (ANOVA) was used to compare variables among the three groups. If the ANOVA was significant, the Bonferroni procedure was used for multiple comparisons. Normally distributed variables were compared using Student's *t*-test and non-normally distributed variables were compared using the Mann–Whitney *U*-test. The Wilcoxon signed rank test was used to compare the degree of infiltration by $IgG4^+$ plasma cells. Correlations were expressed by the Spearman rank correlation coefficient. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS software version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Identification of immunoglobulin G4-associated autoimmune hepatitis

All the AIH patients in this study met the international criteria for the diagnosis of definite AIH. All the AIH patients were negative for serum antibodies against liver–kidney microsome 1 and were diagnosed as type I. Patients who did not meet the international criteria were excluded from the study. These data are shown in Table 1. Liver specimens were obtained from the AIH patients before starting steroid therapy.

Immunoglobulin G4 immunostaining of the liver tissues was performed to assess the degree of infiltration by IgG4-producing plasma cells. We counted the number of $IgG4^+$ cells in at least three portal tracts and calculated the average number of $IgG4^+$ cells in each specimen. We regarded the specimens as IgG4 positive when more than five $IgG4^+$ plasma cells were identified per HPF according to the report by Zhang *et al.* (23). As shown in Figure 1A and B, nine of the 26 AIH patients (34.6%) showed positive staining for IgG4 and were classified into IgG4-associated AIH. In contrast, all 10 patients with PBC, three patients with PSC and 20 patients with HCV hepatitis were negative.

We also examined the IgG4 expression in the livers of two patients with IgG4-associated cholangitis as a positive control. As shown in Figure 1B, the liver sample is heavily infiltrated by $IgG4^+$ cells as compared with the samples from the IgG4-associated AIH patients.

Clinical profile of immunoglobulin G4-associated autoimmune hepatitis

As shown in Figure 2, there were no significant differences in age, serum levels of ALT, ALP, γGTP, antinuclear antibody or in the degree of liver fibrosis between the IgG4-associated and the IgG4 non-associated AIH patients. The IgG4-associated AIH patients had significantly higher total serum IgG levels and AIH scores as compared with the IgG4 non-associated patients. In contrast, there was no difference in serum IgG4 levels between the IgG4-associated AIH and the IgG4 non-associated AIH patients. In addition, we did not find a difference in any of the factors in the AIH scoring system, except for the total serum IgG levels.

Histological analysis of immunoglobulin G4-associated autoimmune hepatitis

We performed an extensive histological analysis of the liver samples taken from the IgG4-associated AIH patients using haematoxylin and eosin-stained tissue. As shown in Figure 3A, portal inflammation and interface hepatitis were present in all liver samples from patients with either IgG4-associated or IgG4 non-associated AIH. In contrast, plasma cell infiltration and lobular hepatitis were detected more frequently in the livers of IgG4-associated AIH than in those of IgG4 non-associated AIH. Although portal inflammation was seen in all the liver samples from AIH patients, the degree of inflammation was more severe in the patients with IgG4-associated AIH (Fig. 3B). Most of the AIH patients were negative for cholangitis on histological analysis. In addition, none of the AIH patients showed any abnormalities of the bile duct as assessed by ultrasonography or computed tomography.

Immunohistochemical analysis of immunoglobulin G4-associated autoimmune hepatitis

Infiltration of T cells, B cells and plasma cells was examined by immunostaining for CD3, CD20, CD38 and IgG using semiconsecutive sections taken from the livers of AIH patients. As shown in Figures 4 and 5, the numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ cells in the livers of patients with IgG4-associated AIH were greater than those in IgG4 non-associated AIH patients. Therefore, IgG4-associated AIH is characterized by the infiltration of T cells, B cells and plasma cells into the liver tissue of these patients. No difference was seen in the number of $IgG1^+$ cells or the ratio of $IgG1^+/IgG^+$ cells between the two subgroups,

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Table 1. Clinical characteristics of patients at the time of diagnosis

	AIH ($n=26$)	PBC ($n=10$)	HCV ($n=20$)
Age (years)	60 ± 9 (42–78)	57 ± 13 (28–72)	56 ± 11 (29–78)
Gender (female/male; %female)	24/2; 92%	9/1; 90%	10/10; 50%*
Concurrent autoimmune diseases	7/26; 27%	0/10; 0%	1/20; 5%
Laboratory data			
AST (IU/L)	196 ± 604 (48–2350)	65 ± 56 (25–210)	58 ± 49 (35–240)†
ALT (IU/L)	216 ± 510 (57–1776)	93 ± 78 (28–234)‡	78 ± 55 (38–229)†
ALP (IU/L)	558 ± 274 (191–1126)	857 ± 269 (480–1217)	244 ± 128 (147–663)†,§
Total bilirubin (mg/dl)	0.9 ± 5.3 (0.4–20)	0.5 ± 0.5 (0.4–2.1)	0.7 ± 0.2 (0.5–1.1)
Albumin (g/dl)	3.9 ± 0.6 (2.3–4.7)	4.0 ± 0.2 (3.7–4.3)	4.1 ± 0.3 (3.3–4.8)†
Immunoglobulin G (g/dl)	2.2 ± 0.7 (1.1–4.1)	1.4 ± 0.8 (1.2–3.9)	1.8 ± 0.7 (0.9–2.8)
IAHG score	19 (16–22)	–	–

Data are expressed as median ± standard deviation (range) or frequency.
*$P < 0.05$ (Fischer's exact test).
†,‡,§$P < 0.05$ between AIH and HCV, between AIH and PBC, and between PBC and HCV respectively.
AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IAHG, International Autoimmune Hepatitis Group; PBC, primary biliary cirrhosis.

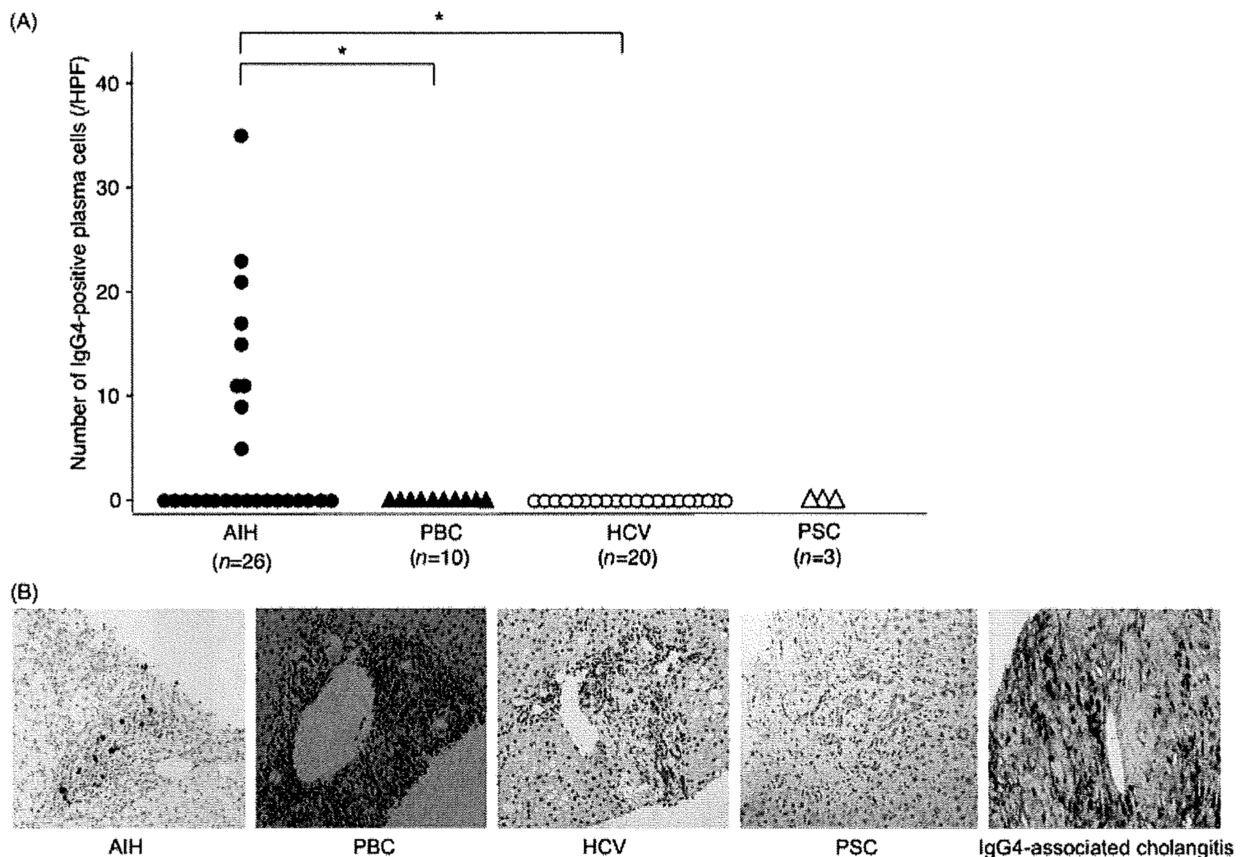

Fig. 1. Identification of immunoglobulin (Ig) G4-associated autoimmune hepatitis (AIH). (A) Number of IgG4$^+$ plasma cells/high-power field in liver specimens from 26 patients with AIH, 10 patients with primary biliary cirrhosis (PBC), three patients with primary sclerosing cholangitis (PSC) and 20 patients with hepatitis C virus (HCV) hepatitis. Nine of the 26 (34.6%) samples from the AIH patients showed positive staining for IgG4. All 10 patients with PBC, 20 patients with HCV and three patients with PSC were negative. *$P < 0.05$. (B) Representative sections showing the immunohistochemical staining of IgG4 in the livers of patients with IgG4-associated AIH, PBC, HCV hepatitis, PSC and IgG4-associated cholangitis.

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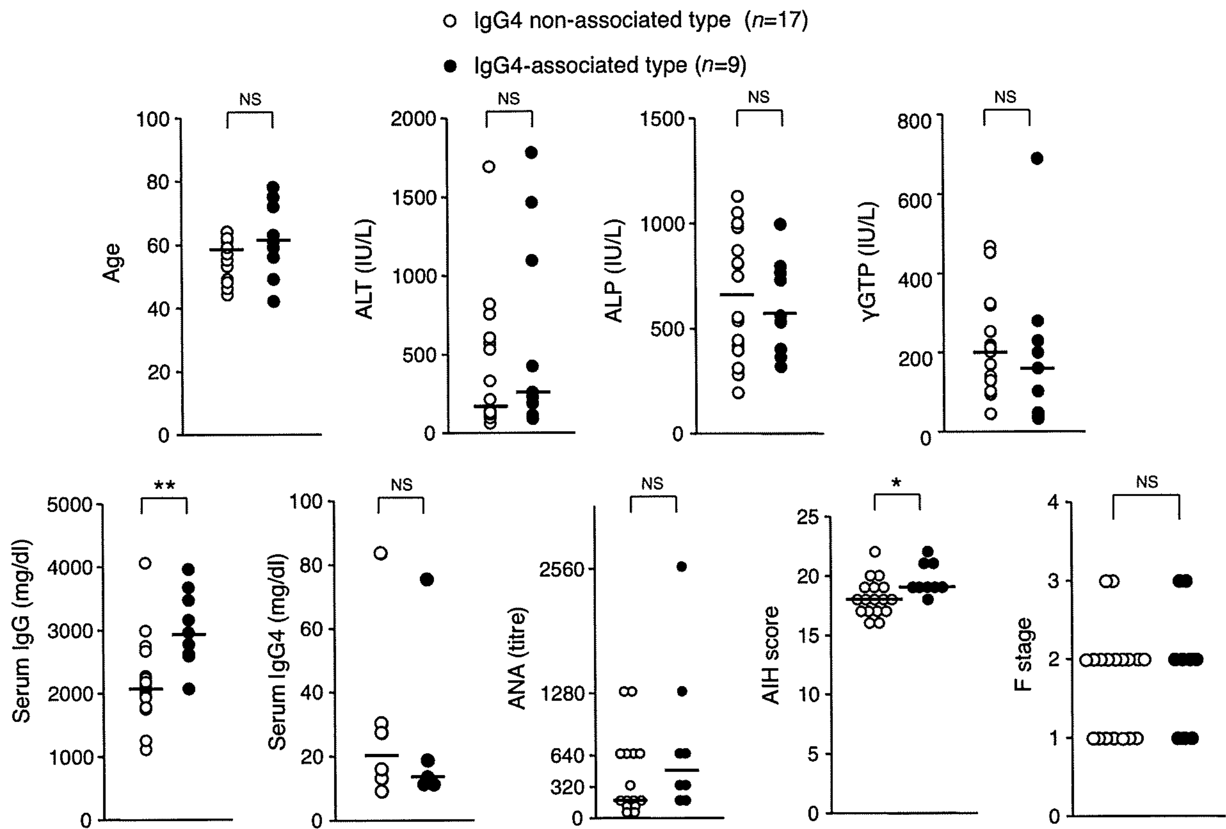

Fig. 2. Comparison of the clinical characteristics between immunoglobulin (Ig) G4-associated and IgG4 non-associated autoimmune hepatitis (AIH). Horizontal bars represent median values in each plot. Serum IgG levels and AIH scores were significantly higher in IgG4-associated AIH patients than in IgG4 non-associated AIH patients (*$P < 0.05$, **$P < 0.01$). NS, not significant.

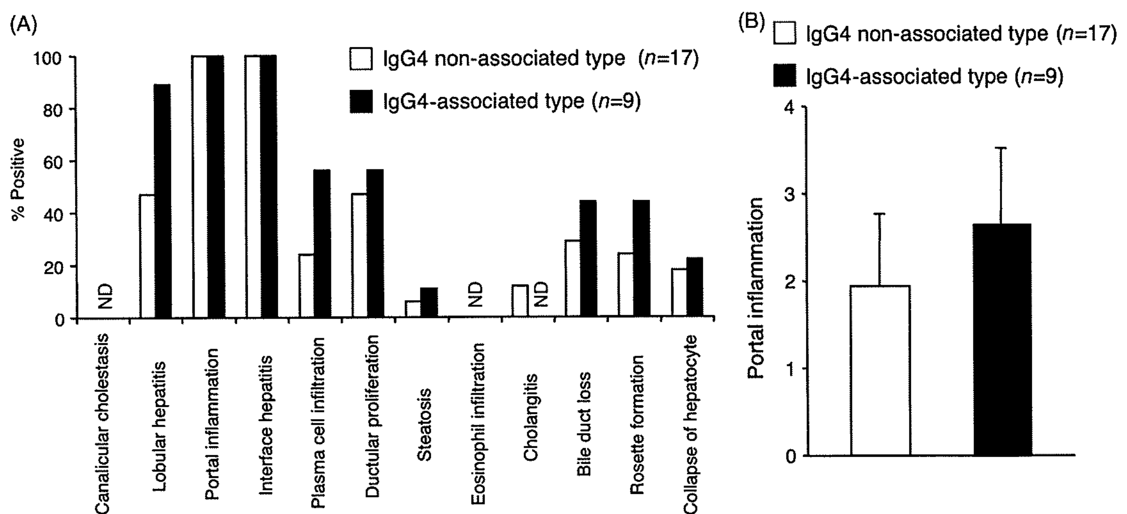

Fig. 3. Comparison of the histological findings between immunoglobulin (Ig) G4-associated and IgG4 non-associated autoimmune hepatitis (AIH). (A) Haematoxylin and eosin-stained liver tissues obtained from AIH patients were analysed. Plasma cell infiltration and lobular hepatitis were more frequently detected in the livers of IgG4-associated AIH patients than in IgG4 non-associated AIH patients, whereas portal inflammation and interface hepatitis were seen in all liver samples. ND, not detected. (B) The degree of portal inflammation as assessed by the Ishak scoring system.

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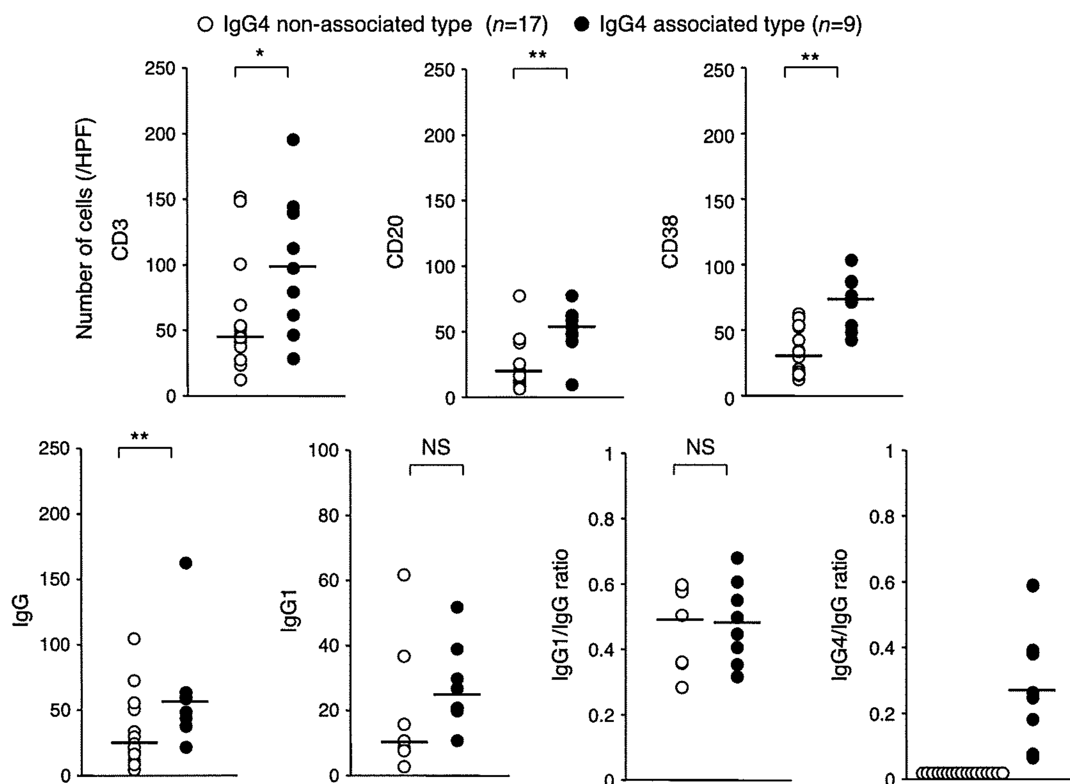

Fig. 4. Numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells, immunoglobulin (Ig) G^+ cells and $IgG1^+$ cells in the liver specimens from patients with autoimmune hepatitis (AIH). Horizontal bars represent median values in each plot. The numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ cells were greater in the livers of patients with IgG4-associated AIH than in patients with IgG4 non-associated AIH ($^*P < 0.05$, $^{**}P < 0.01$). NS, not significant.

suggesting a selective accumulation of $IgG4^+$ cells in IgG4-associated AIH. No correlation was seen between the number of $IgG4^+$ plasma cells and the number of $CD3^+$ T cells, $CD20^+$ B cells or $CD38^+$ plasma cells in the livers of patients with IgG4-associated AIH (data not shown). Moreover, the number of $IgG4^+$ plasma cells did not correlate with the AIH score or the serum ALT level, suggesting that the number of $IgG4^+$ cells is not associated with the severity of AIH (data not shown).

Response to steroid therapy in patients with immunoglobulin G4-associated autoimmune hepatitis

Finally, we compared the response of IgG4-associated and IgG4 non-associated AIH patients to steroid therapy. Six patients with IgG4-associated AIH and 16 patients with IgG4 non-associated AIH were treated with prednisolone at an initial dose of 30–40 mg/day, followed by maintenance therapy at 5–10 mg/day (Fig. 6). No difference was seen in the treated dose of prednisolone between IgG4-associated and IgG4 non-associated AIH patients. As shown in Figure 6, a significant decrease of serum ALT levels was seen in patients with IgG4-associated AIH and those with IgG4 non-associated AIH at 4 weeks after starting prednisolone therapy. Importantly, the reduced serum ALT levels in IgG4-associated AIH were maintained at 48, 72 and 96 weeks after starting prednisolone therapy. This was not the case for the IgG4 non-associated AIH patients. None of the patients with IgG4-associated AIH showed elevated levels of serum ALT on prednisolone therapy. In contrast, eight patients (50.0%) with IgG4 non-associated AIH did show elevated levels of serum ALT, even on prednisolone therapy. Furthermore, no relapse was seen in the patients with IgG4-associated AIH, whereas six patients with IgG4 non-associated AIH (37.5%) required incremental doses of prednisolone because of relapse. Thus, IgG4-associated AIH shows a marked response to prednisolone not only in the initial phase but also in the maintenance phase of treatment.

Discussion

In this study, we describe a novel subtype of AIH characterized by infiltration of IgG4-expressing plasma cells. Our data show that infiltration by $IgG4^+$ plasma cells does occur in a subpopulation of AIH patients but not in PBC or PSC patients. Thus, tissue staining for IgG4 can be used to classify AIH into either an IgG4-associated type or an IgG4 non-associated type. Patients with IgG4-associated AIH have increased levels of serum IgG and AIH scores as compared with patients with the

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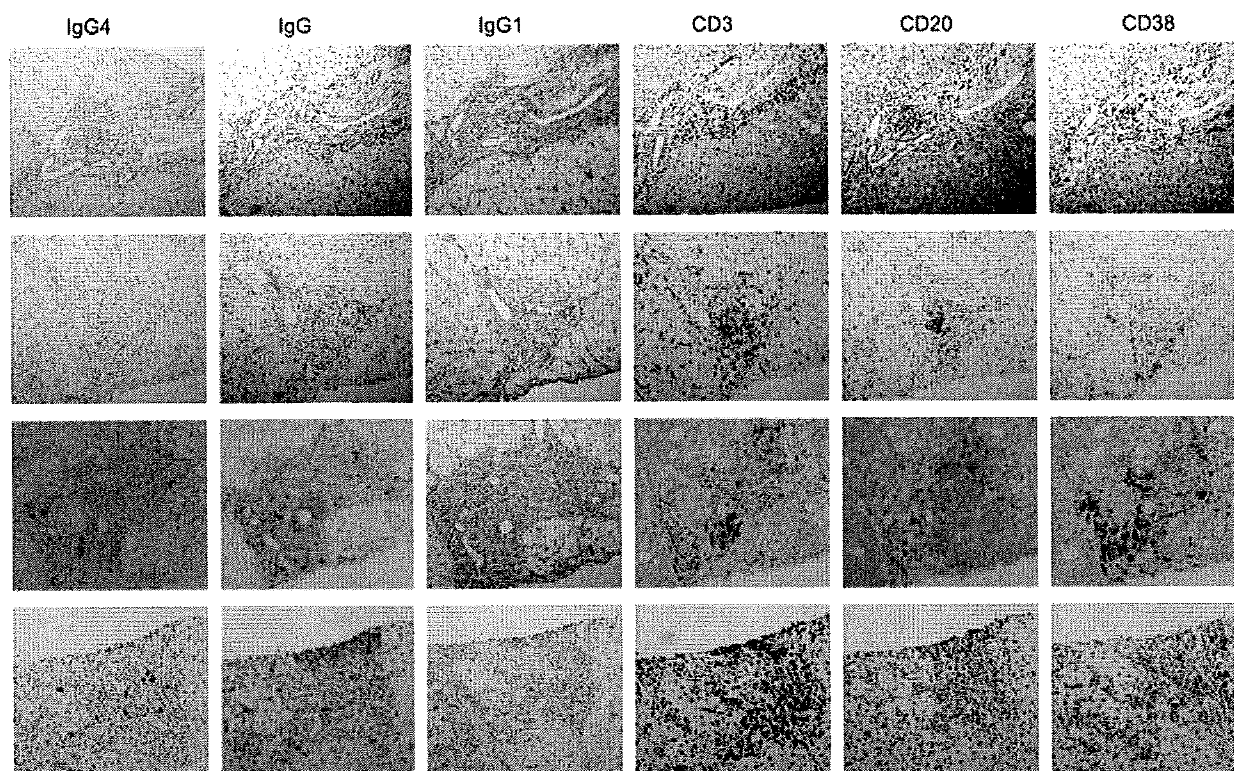

Fig. 5. Immunohistochemical staining of immunoglobulin (Ig) G4, IgG, IgG1, CD3, CD20 and CD38 in the livers of patients with autoimmune hepatitis (AIH). The top two and bottom two panels show liver sections from IgG4 non-associated and IgG4-associated AIH patients respectively. The numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ plasma cells in the livers of the patients with IgG4-associated AIH were greater than in those of the patients with IgG4 non-associated AIH.

IgG4 non-associated type. More importantly, prednisolone therapy is very effective in patients with IgG4-associated AIH for both induction and maintenance of remission. We regarded the tissue specimens as IgG4 positive when more than five $IgG4^+$ plasma cells were identified per HPF as reported by Zhang *et al.* (23), although significant differences in the response to prednisolone and in serum IgG levels were still observed when we set a positive threshold of 10 $IgG4^+$ plasma cells per HPF as reported by Ghazale *et al.* (13) (data not shown). Our data suggest that positive IgG4 staining in the liver can be used as a surrogate marker for the subtype of AIH that responds well to prednisolone therapy.

Some of the patients with IgG4-related AIP have various extrapancreatic lesions. Because these extrapancreatic and pancreatic lesions share common histopathological findings (i.e. abundant infiltration by $IgG4^+$ plasma cells), Kamisawa and Okamoto (10) proposed a new clinicopathological entity: 'IgG4-related sclerosing disease'. Portal inflammation and lobular hepatitis characterized by abundant infiltration of $IgG4^+$ plasma cells is often seen in the livers of AIP patients (12, 15). This AIP-associated liver inflammation is called IgG4 hepatopathy (15). One important issue arising from our study is whether IgG4-associated AIH is a hepatic manifestation of this systemic disease rather than a subtype of classical AIH. It should be noted that none of the AIH patients in this study showed any swelling of the pancreas or abnormality of the bile or pancreatic ducts as assessed by ultrasonography or computed tomography findings that are characteristic of IgG4-associated AIP and cholangitis (7). In addition, extrapancreatic manifestations of IgG4-related systemic disease such as sialadenitis, retroperitoneal fibrosis and inflammatory liver pseudotumour (10) were absent in all AIH patients studied. More importantly, serum IgG4 levels in patients with IgG4-associated AIH were not so high as those in patients with IgG4-related systemic disease. These results suggest that IgG4-associated AIH is distinct from IgG4-related systemic disease, including AIP and IgG4 hepatopathy, although all show marked responses to prednisolone therapy. Further examination of the pancreatobiliary system using endoscopic retrograde cholangiopancreatography or magnetic resonance cholangiopancreatography may be useful to confirm that IgG4-associated AIH is not a hepatic manifestation of IgG4-related systemic disease including IgG4-associated cholangitis.

Although enhanced IgG4 antibody responses are linked to AIP, the role of this antibody in disease development and progression is poorly understood (7). Enhanced IgG4 antibody responses may be an epiphenomenon associated with inflammatory reactions (7). One possibility is that

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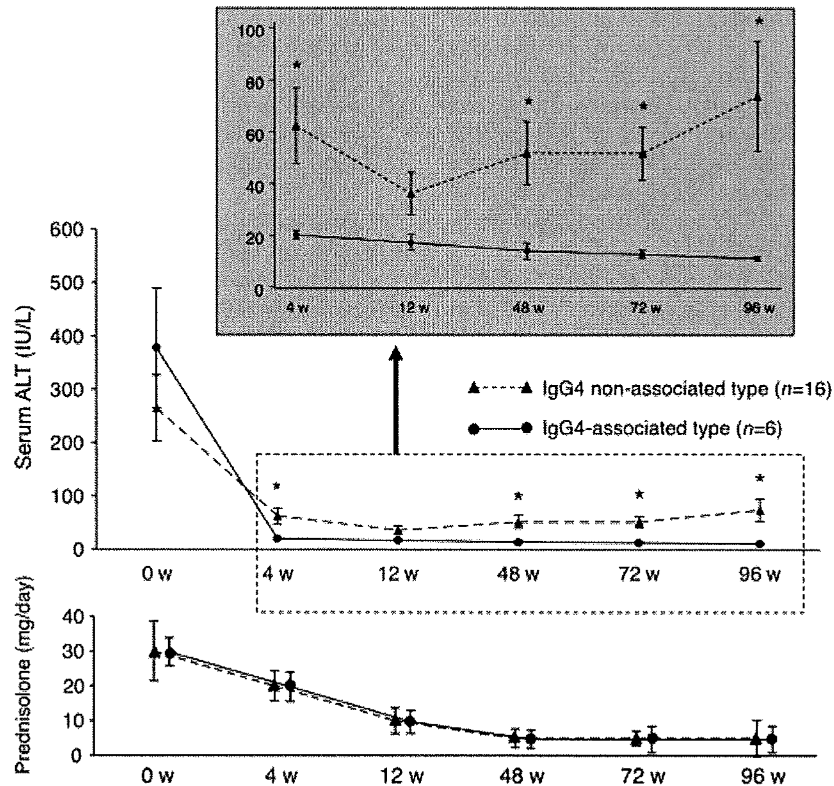

Fig. 6. Serial changes of serum alanine aminotransferase (ALT) levels in patients with autoimmune hepatitis (AIH) during treatment with prednisolone. Serum levels of ALT were monitored in six patients with immunoglobulin (Ig) G4-associated AIH and in 16 patients with IgG4 non-associated AIH. These patients were treated with prednisolone. The doses of prednisolone at each time point are shown in the bottom panel. The results are shown as mean ± standard error ($^*P < 0.05$).

the increase in tissue-infiltrating IgG4$^+$ plasma cells in IgG4-associated AIH simply reflects the degree of migration and accumulation of B cells and IgG$^+$ plasma cells. In fact, we found that the numbers of CD20$^+$ B cells and CD38$^+$ plasma cells are greater in the livers of IgG4-associated AIH patients than in those of IgG4 non-associated AIH patients. However, we found no difference in the number of IgG1$^+$ cells or in the ratio of IgG1$^+$/IgG$^+$ cells between the two subgroups. Although the possibility of a non-selective increase in the migration and accumulation of B cells cannot be completely excluded, the results of IgG1 expression suggest that selective augmentation of IgG4 production occurs in the livers of patients with IgG4-associated AIH. Therefore, the involvement of factors driving IgG4 class switching may be considered in the immunopathogenesis of IgG4-associated AIH. Further studies are necessary to elucidate the molecular mechanisms for the selective accumulation of IgG4$^+$ plasma cells in the liver.

Serum levels of IgG4 were not increased in patients with IgG4-associated AIH. This finding suggests that tissue staining of IgG4 is more sensitive than serum IgG4 assays for the diagnosis of IgG4-associated AIH. In support of this, Zhang *et al.* (23) reported that visualization of IgG4$^+$ plasma cells is useful for the diagnosis of AIP in patients with normal levels of serum IgG4. However, Umemura *et al.* (14) reported the case of IgG4-associated AIH with a marked elevation in serum IgG4 levels. The reason for this discrepancy remains unknown. It should be noted that Umemura's case was characterized by liver infiltration by many IgG4$^+$ cells (> 40/HPF). This is much higher than we observed in any of our cases. This suggests that it is the degree of accumulation of IgG4$^+$ cells in the liver that determines the serum IgG4 response in patients with IgG4-associated AIH. Another possibility is that immune environments, which affect IgG4 responses, may be different in the peripheral blood and the liver of patients with IgG4-associated AIH. Indeed, predominant Th1 responses have been reported in the peripheral blood of patients with AIP (24), whereas Th2 responses are enhanced in the pancreas and liver (25). However, further studies using a large number of AIH patients are necessary to establish the relationship between serum IgG4 levels and IgG4 expression in the liver.

Our data show that IgG4$^+$ cells are distributed in the liver of IgG4-associated AIH in a scattered manner rather than in a densely packed manner. In addition, there is no difference in serum IgG4 levels between the IgG4-associated AIH and the non-associated AIH patients. More

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importantly, the number of AIH patients enrolled in this study may not be sufficient to enable definite conclusions to be drawn. Thus, one might concern that the difference between these two groups is small. However, our results clearly show that the effectiveness of steroid therapy was different in the two groups of patients (Fig. 6). Future studies using a larger number of AIH patients would be required to confirm the difference between IgG4-associated AIH and non-associated AIH.

Patients with classical AIH usually show marked responses to steroid therapy (1). We have shown that the serum levels of ALT were reduced in most AIH patients 4 weeks after starting prednisolone treatment. An interesting observation is that the reduction in the serum ALT level in the IgG4-associated AIH was greater than that in the IgG4 non-associated AIH. Furthermore, remission was maintained in the patients with IgG4-associated AIH. Therefore, IgG4-associated AIH is characterized by a marked response to prednisolone treatment similar to that seen in patients with IgG4-related systemic disease (26). We found no relapse in patients with IgG4-associated AIH, whereas six patients with IgG4 non-associated AIH required incremental doses of prednisolone because of a relapse.

In conclusion, our data suggest that the immunostaining of liver biopsy specimens for IgG4 can be used to predict the prognosis of AIH patients by identifying the subpopulation that shows a marked response to steroid treatment. Further studies using larger patient groups are required to confirm these findings.

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No conflicts of interest exist.

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TOPICS
Hepatocellular carcinoma: Eastern and Western experiences

Single HCC between 2 and 5 cm: the grey zone

Hepatologist's perspective

Masatoshi Kudo · Hobyung Chung

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Abstract Long term survival of single nodular hepatocellular carcinoma (HCC) measuring 2–5 cm in size treated with radiofrequency ablation (RFA) was compared between 157 HCCs treated with RFA and 89 HCCs treated by operation. The 5 year survival rate was not significantly different between RFA and operation. In addition, the 17th nationwide survey of the Liver Cancer Study Group of Japan clearly showed that 5 year survival rate in 858 HCCs measuring 2–5 cm in size treated with RFA was similar to that in 6,574 HCCs measuring 2–5 cm in size treated by operation. In conclusion, RFA achieves outcomes similar to those of operation for Child-Pugh A patients with HCCs measuring 2–5 cm in size.

Keywords Hepatocellular carcinoma · Radiofrequency ablation · Surgery · Single HCC between 2 and 5 cm

Introduction

Hepatocellular carcinoma (HCC) is a poor-prognostic disease, and nearly one million people die of this cancer annually worldwide. Various treatments have been proposed as candidates for a therapeutic policy for HCC. The guidelines established by the American Association for the Study of Liver Diseases (AASLD) [1], European Association for the Study of the Liver (EASL) [2] and the Japanese "Evidence-based guidelines" [3] recommend local treatment, mainly radiofrequency ablation (RFA), for 3 or fewer HCCs measuring 3 cm or smaller. Only the "Treatment algorithm for HCC" proposed by the Japan Society of Hepatology (JSH) recommends combination therapy with transcatheter arterial chemoembolization (TACE) followed by RFA for non-resectable 3-cm or larger HCCs [4] (Fig. 1).

In this report, we present the outcomes of treatments performed according to this algorithm at our hospital as well as those obtained by the Liver Cancer Study Group of Japan (LCSGJ).

Subjects and methods

The subjects were 157 patients with Child-Pugh A liver function and a 5-cm or smaller solitary HCC who underwent RFA or RFA preceded by TACE at the Department of Gastroenterology and Hepatology, Kinki University School of Medicine, between 1999 and 2007. There were 122 males and 50 females aged 47–90 years (mean: 68 years), and 20 were HBV-positive, 129 were HCV-positive, and 8 were virus-negative. The tumor size was 10–50 mm (mean: 19 mm): 85 and 72 nodules measured 2 cm or smaller and 2–5 cm, respectively. The number of RFA sessions was 1–3 (mean: 1.2), and the mean duration of follow-up was 40.3 months. The number of subjects who underwent surgery during the same period was 89.

RFA was performed by using cooled tip needle (Radionics, Burlington, MA) and preceding TACE was performed basically in cases the tumors were larger than 2 cm. Lipiodol mixed with epirubin followed by gelatine sponge was used for Lipiodol TACE. Enough safety margin (ablative margin) was obtained by ablating an area larger than the Lipiodol deposited tumor. Overall survival

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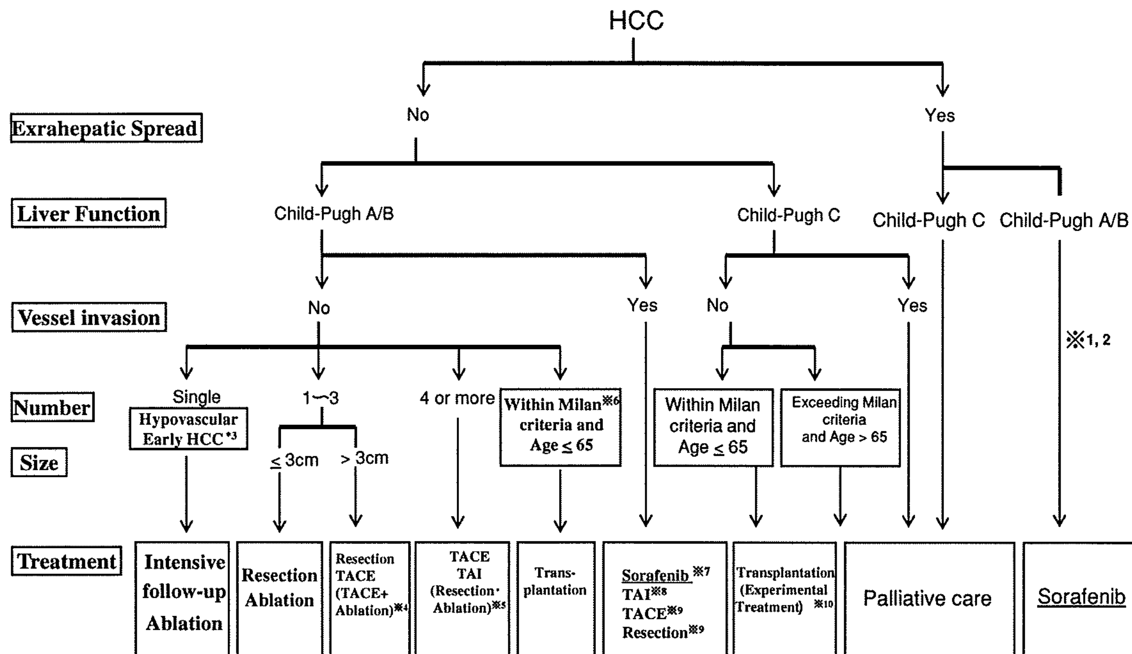

Fig. 1 Consensus-based treatment algorithm for hepatocellular carcinoma proposed by Japan Society of Hepatology (JSH). Cited from [4]

of the patients who underwent RFA± preceding TACE with a solitary tumor smaller than 2 cm or less in size and those of 2–5 cm in size was evaluated.

Similarly, overall survival of the Child-Pugh A patients with solitary 2–5 cm sized HCC who underwent resection was compared with that of the patients who underwent RFA combined with preceding TACE. Local recurrence rate and disease-free survival rate were also calculated in Child-Pugh A patients with 2–5 cm sized HCC who underwent RFA combined with preceding TACE. The relationship between recurring segment and overall survival was also evaluated in Child-Pugh A patients with 2–5 cm sized HCC who underwent RFA combined with preceding TACE. Risk factors affecting overall survival were also evaluated in patients who underwent RFA combined with preceding TACE.

In order to identify the difference of the overall survival in Liver damage A (almost equivalent to Child-Pugh A) patients with a solitary 2–5 cm HCC, survival data of the 17th nationwide survey by the Liver Cancer Study Group was used [5]. For HCCs of 2–5 cm, resection, PEIT and RFA was performed in 6,574 patients, PEIT in 2,823 patients and RFA in 858 patients. Overall survival was compared among the 3 groups.

Results

The 5-year survival rate was 79% in the 2-cm or smaller HCC group treated with RFA and 84% in the 2–5 cm

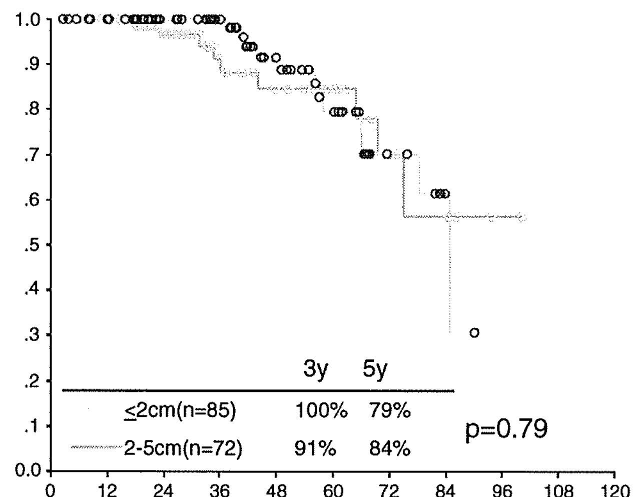

Fig. 2 Overall survivals of Child-Pugh A HCC patients carriers of single HCC smaller than 2 cm in size, and treated with RFA and of Child-Pugh A HCC patients carriers of single HCC 2–5 cm in size treated by RFA combined with preceding TACE

HCC group treated with TACE preceding RFA, showing no significant difference (Fig. 2). The latter, when compared with 89 patients who underwent surgery also for single HCC 2–5 cm in size, did not show statistically different survivals (84 vs. 78% respectively) (Fig. 3). Among the 157 cases with a single HCC 2–5 cm, overall local recurrence occurred in 10, and the 7-year cumulative local recurrence rate was 8%. The 5-year disease-free

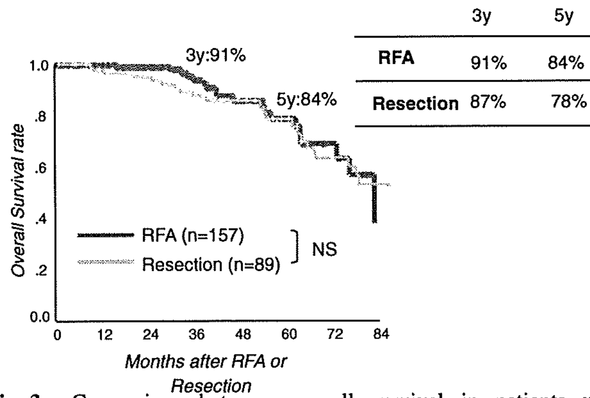

Fig. 3 Comparison between overall survival in patients who underwent resection and RFA for solitary HCC smaller than 5 cm in Child-Pugh A cirrhosis

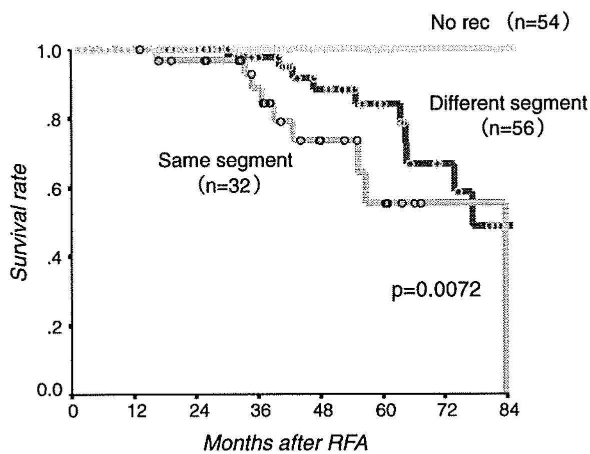

Fig. 4 Overall survival rate for patients carriers of single HCC 2–5 cm in size in Child-Pugh A cirrhosis and treated with RFA combined with preceding TACE grouped according to the site of the recurrence

survival rate of patients with 2 to 5-cm solitary HCC was 15%. Therefore, the 5-year cumulative recurrence rate was as high as 85%. The recurrent cases were divided into 32 cases with recurrence localized in the same segment of the primary lesion and 56 cases with recurrence in different segments. The prognosis was significantly poorer in those with recurrence in the same segment than in those in those with new lesions in different segments (Fig. 4).

A significant prognostic factor in the Child-Pugh A single nodular HCC cases with recurrence in the same segment was as follows: (1) a macroscopic type other than single nodular type versus single nodular with extra growth type or multinodular confluent type, (2) a PIVKA-II level of 100 mAU or higher and (3) AFP level of 100 ng/ml or higher. On multivariate analysis of factors contributing to the overall survival, the macroscopic type, AFP, and PIVKA-II were extracted as significant factors. Factors contributing to disease-free survival were only AFP and PIVKA-II, but there were no significant differences between HCCs smaller than 2-cm or those 2–5 cm (Fig. 5).

Results of the 17th nationwide survey performed by the Liver Cancer Study Group of Japan

Figure 6 shows the survival curves of patients who underwent surgery, PEIT and RFA for single 2 to 5-cm HCCs, respectively [5]: the prognosis was significantly poorer in the PEIT group (Fig. 6). Conversely, outcomes were similar comparing those treated with surgery and those with RFA for single 2–5 cm HCCs (Fig. 6) [4].

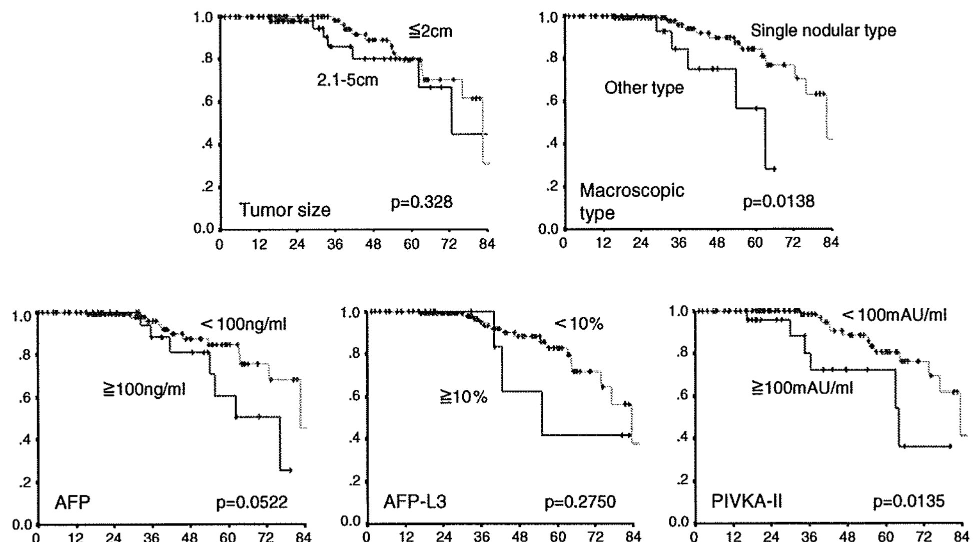

Fig. 5 Factors affecting overall survival in patients with Child-Pugh A liver function and 2–5 cm tumors who underwent RFA combined with preceding TACE