Table 4 Relationship among clinicopathologic findings, HCV infection, and *Bcl* genes of frozen tumor tissues

	Age (y)	Sex	CG	Serum HCV		Hepatic disorder	Primary tumor site	Histology	HCV RNA copy per 10 mg	Immunohistology			Rearrangement of *Bcl* genes		Immunohistology		
				Ab	Genotype					HCV NS3	HCV E2	CD81	*Bcl2*	*Bcl6*	CD10	Bcl2	Bcl6
1	60	M	–	+	1b	CH	Spleen	DLBCL	4.1×10^2	+	+	+	–	–	–	–	+
2	59	M	–	+	1b	CH	Spleen	DLBCL	2.5×10^2	+	+	+	–	–	–	+	+
3	69	M	–	+	2b	CH	Spleen	DLBCL	1.9×10^5	+	+	+	–	+	–	–	+
4	59	F	NT	+	NT	CH	Spleen	DLBCL	4.0×10^5	+	+	+	–	–	–	+	–
5	49	M	NT	+	1b	CH	Spleen	DLBCL	5.5×10^4	–*	–*	+	–	–	–	+	+
6	53	F	NT	+	NT	CH	Spleen	DLBCL	1.4×10^3	+	+	+	–	+	+	–	+
7	67	M	NT	+	NT	CH	Spleen	DLBCL	0	–	–	+	–	–	–	–	+
8	71	F	NT	+	1b	CH	Spleen	Marginal	0	–	–	+	–	–	–	+	–
C1	69	F	–	+	1b	CH	LN	Reactive	9.5×10^3	–*	–*	–**	–	+	R+	R+	R+
C2	61	M	–	+	1b	CH	LN	Reactive	2.9×10^2	NT	NT	NT	–	–	R+	R+	R+
C3	74	M	–	+	1b	CH	LN	Reactive	8.0×10^4	NT	NT	NT	NT	NT	R+	R+	R+

Abbreviations. Ab, antibody; M, male; F, female; LN, lymph node; R, reactive changes.
* HCV NS3- or E2-positive small lymphocytes are found.
** CD81-positive histiocytes are present.

86% by the Kaplan-Meier method, and that of HV-negative cases was 100%.

3.3. Immunohistological findings and EBV infection in splenic DLBCL

Immunohistologically, 6 (31%) of 29 DLBCL cases showed a positive reaction for CD10, and 9 (37.5%) of 24 were positive for CD25. *Bcl6*-positive lymphoma cells were found in 16 (66.7%) of 24 cases, and *Bcl2* was found in 11 (45.8%). No positive reaction for HHV-8 LNA was detected in any of the 24 examined DLBCL cases. By in situ hybridization, only 1 of 24 examined DLBCL cases possessed EBER-positive tumor cells (Table 3).

3.4. Other types of lymphoma primarily involving the spleen

One of the 10 SMZL cases showed HCV infection, and 2 others showed HBV infection. Nine cases received a

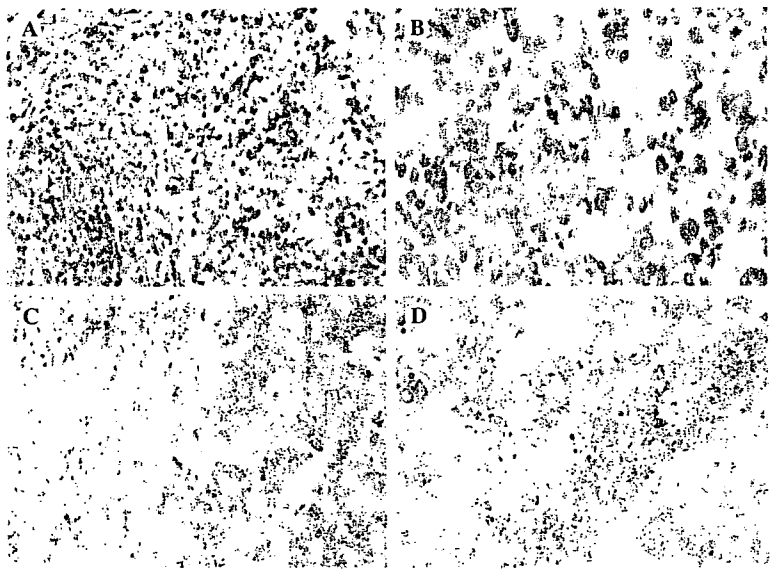

Fig. 2 A, Immunohistology of HCV NS3 protein in the spleen. Some scattered reactive lymphocytes are detected in the red pulp outside the DLBCL (avidin-biotin complex method, original magnification ×200). B, A high-magnification image of HCV NS3-positive reactive lymphocytes (original magnification ×400). C, Immunohistology of HCV NS3 protein in DLBCL. Large tumor cells show an intracytoplasmic reaction for HCV NS3 protein (original magnification ×300). D, Immunohistology of the HCV E2 protein in DLBCL. Many tumor cells are positive for the HCV E2 protein (original magnification ×400).

splenectomy, and the mean weight of the removed spleen in these cases was 1610 g, which was significantly ($P < .01$) heavier than that of the DLBCL cases. Numerous white miliary nodules were detected in the whole spleens, and no large tumor formations were detected (Fig. 1B and C). Six cases were classified as stage III or IV, and combined chemotherapy was performed in 10 cases. Only 1 case without HV infection died of disease at 34 months. Three cases of splenic mantle cell lymphoma had no HV infection. Among 13 cases of B-CLL, 1 case was positive for the HBsAg and HBeAg. One case of CD25- and CD103-positive hairy cell leukemia was HBsAg- and HCV antibody–negative. In 12 HST/NKL cases, only 1 aggressive NKL was HCV antibody–positive.

3.5. Detection of HCV RNA, HBV DNA, and HV antigens

The detailed findings for tissue HCV RNA and immunohistology of HCV antigens are shown in Table 4. Of 7 DLBCL cases with the HCV antibody in their sera, 6 showed between 1.4×10^3 and 4.0×10^4 copies of HCV RNA per 10-mg tumor tissues by real-time PCR. In 1 SMZL case showing the HCV 1b genotype in the serum, HCV RNA was not detected in the involved tissue. HCV RNA was detected in the reactive lymph nodes of 3 HCV-positive cases. Immunohistologically, weak positive reactions for the HCV NS3 and E2 proteins were detected in the cytoplasm of many lymphoma cells in 5 of 7 DLBCL cases (Fig. 2) but not in 1 SMZL case. Outside the tumors, some HCV NS3– and E2 protein–positive reactive lymphocytes were detected, mainly in the marginal zone and red pulp (Fig. 2). Lymphoma cells and scattered histiocytes were positive for CD81 in 7 DLBCL cases. Among these cases, the lymphoma cells in 2 HCV protein–negative cases showed a positive reaction for CD81. Two examined DLBCL cases showed 1.8×10^3 and 1.6×10^4 copies of HBV DNA per 10-mg tumor tissues by real-time PCR. Immunohistologically, the large lymphoma cells in 2 of 6 DLBCL cases showed a weak nuclear reaction for the HBs protein in the formalin-fixed tumor tissues.

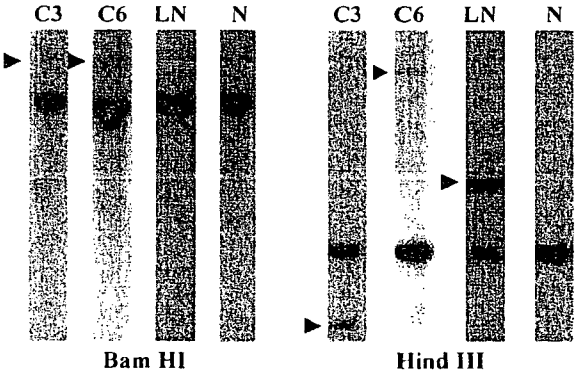

Fig. 3 Southern blot analysis of *Bcl6* genes. Genomic DNAs of fresh frozen tissues are digested with *Bam*HI or *Hin*dIII. Cases 3 and 6 of HCV-positive splenic DLBCL and a control case of HCV-positive reactive lymph node show rearrangements of *Bcl6* genes. Genomic DNA of a HCV-negative reactive tonsil is used as a negative control. NOTE. Arrowheads indicate rearranged bands. Abbreviations. C3, case 3; C6, case 6; LN, control case of HCV-positive reactive lymph node; N, negative control.

3.6. Examination of the Bcl2 and Bcl6 genes and proteins

These data are also shown in Table 4. No rearrangement of the *Bcl2* gene was detected in the fresh tumor tissues of 7 DLBCL cases, whereas 2 DLBCL cases and 1 reactive lymph node showed rearrangement of the *Bcl6* gene (Fig. 3). Immunohistologically, 3 of 7 DLBCL cases showed a positive reaction for the *Bcl2* protein and 6 were positive for *Bcl6*.

4. Discussion

Saadoun et al [18] demonstrated that treatment with IFN alone or a combination of IFN and ribavirin led to a complete virologic response and hematologic remission as well as the disappearance of type II CG and its clinical symptoms in 16 of 18 examined HCV-positive SMZL cases. The monoclonal IgM of type II CG, which is frequently (17.3%) found in HCV-positive patients [19], is mostly encoded by a restricted set of immunoglobulin V region genes, *VH1-69* and *Vκ3-A27*, which are also expressed in more than half the cases of HCV-positive LPL and salivary gland MALToma [20,21]. It has been suggested that HCV-positive type II CG is a prodromal phase in cases of SMZL, LPL of the bone marrow, and salivary gland MALToma in southern Europe. The HCV prevalence (2.5%) in blood donors of 50 to 64 years in Japan was similar to that (2.4%) in blood donors older than 40 years in Italy [22], and the HCV genotypes did not differ between Japanese and Italian cases of B-cell ML [3,23]. However, the incidence of type II CG is 1.5% in HCV-positive Japanese cases [24] and 3.5% in HCV-positive B-cell ML [23]. In this study, only 1 (10%) SMZL case had HCV infection, and 4 HCV-positive DLBCL cases and 3 HCV-positive reactive lymphadenitis cases examined had no type II CG. It is strongly speculated that the rare incidence of type II CG and its low association with abnormal B-cell proliferation in HCV-positive cases influence the low incidence of SMZL. Lenzi et al [25] demonstrated that haplotype HLA-B8-DR3 may be considered as a risk factor for developing HCV-positive CG in Italy. However, the haplotype was not found in Japanese HCV-positive cases either with or without CG [26]. Therefore, host genetic factors may influence the incidence of type II CG in HCV-positive cases.

On the other hand, the prevalence (51.7%) of HCV infection in the examined splenic DLBCL cases was significantly ($P < .05$) higher than those in the SMZL and node-based DLBCL cases. Some HCV-positive splenic

DLBCL cases have been reported [3,7], but no detailed analyses of the relationship between splenic DLBCL and HCV infection have been performed. Murakami et al [27] found a high prevalence (14.3%) of chronic liver disease in 98 reported Japanese cases of splenic ML in 1988. Although no adequate virologic examination was performed, they speculated that chronic liver disease has some etiologic influence on splenic ML. Among 15 examined splenic DLBCL cases with HCV infection, 12 had chronic hepatitis or were healthy carriers. HCV infection, which is independent of the degree of hepatic dysfunction, may influence the local immune system and play a role in the lymphomagenesis of splenic DLBCL.

De Vita et al [3] demonstrated that type II CG was only detected in 4 (17.4%) of 23 HCV-positive DLBCL cases and suggested that HCV-positive B-cell ML without type II CG tends to show high-grade B-cell ML [3,28]. Our 4 examined HCV-positive splenic DLBCL cases did not show type II CG. However, 3 of 4 HCV-positive hepatic DLBCL cases were reported to have type II CG in France [29]. Further studies are necessary to examine which kinds of factors influence the occurrence of HCV-positive splenic DLBCL.

Using reverse transcription PCR, Luppi et al [30] detected HCV RNA in tumor tissues from 6 of 8 low-grade MALToma cases, 5 of 8 follicular lymphoma, and only 1 of 14 Hodgkin lymphoma but not in tumor tissues from 10 T-cell lymphoma cases. Immunohistologically, the HCV core and NS3 and NS4 proteins C100, c22, and c33 were mainly detected in lymphocytes in the interfollicular area of reactive lymph nodes and in 3 follicular lymphoma cases among 12 HCV-positive B-cell ML [9]. We also found HCV RNA in tumor tissues from 6 of 7 examined splenic DLBCL cases as well as from 3 reactive lymph node cases by real-time PCR. Furthermore, the HCV NS3 and E2 proteins were detected in the lymphoma cells of 5 of 7 DLBCL cases. It was previously reported that NS3 was able to transform a nonneoplastic mouse fibroblastic cell line into a fibrosarcoma [31]. Hofmann et al [32] demonstrated that the HCV E2 protein may induce B-cell proliferation in vitro and the development of type II CG in vivo. HCV may replicate in B cells and be closely connected with B-cell lymphoma in HCV-positive patients.

CD81 has been identified as a receptor for the HCV E2 protein in B cells [11]. In the current study, CD81 was positive in the lymphoma cells of 7 HCV-positive splenic DLBCL cases and even in those of the 2 HCV E2 protein–negative cases. CD81, which belongs to the tetraspanin family, also has close associations with major histocompatibility complex class I and II proteins [33]. It was also detected in 19 of 21 Burkitt cell lines, which are not connected with HCV infection [34]. Therefore, CD81 may play a role not only as a receptor for HCV E2 protein but also in signaling through major histocompatibility complex molecules in B cells.

Sasso et al [35] reported a high joining rate (82%) of the *Bcl2* gene to immunoglobulin J_H6 in the PBMCs of HCV-positive cases by nucleotide sequence analysis, which was significantly ($P = .03$) higher than the rate for normal controls (38%) and similar to that (66%) of HCV-positive B-cell ML cases. However, Boiocchi [36] demonstrated that *Bcl2* translocation occurred in PBMCs from ML cases independently of HCV infection, and only 4 (12.5%) of 32 cases showed rearrangement of the *Bcl2* gene in the tumor tissue of HCV-positive B-cell ML. These authors suggested that the *Bcl2* gene is not the main inducer of lymphomagenesis in HCV-positive B-cell ML. In our study, 7 splenic DLBCL cases with HCV infection showed no rearrangement of the *Bcl2* gene in their tumor tissues by Southern blot analysis, and only 2 had a rearrangement of the *Bcl6* gene. Mutations of the *VH* and *Bcl6* genes as well as the p53 and β-catenin genes occurred 7-fold more frequently in HCV-positive B-cell lines and ML cases than in HCV-negative controls [37]. Different kinds of mutations in the cell cycle and apoptotic pathway may be required for HCV-positive, low- and high-grade B-cell ML.

Stoll-Becker et al [38] detected different HBV messenger RNA transcripts in PBMCs from carriers using paired comparative PCR, whereas Galun et al [39] demonstrated immunohistologically that the HBsAg was present in lymphoma cells in about 20% of HBV-positive B-cell ML cases. Kim et al [40] reported that the adjusted odds ratios of HBV infection of 136 patients with B-cell ML were 2.42 versus patients with nonhematopoietic disorders and 4.57 versus patients with nonmalignant conditions in South Korea. In the examined splenic DLBCL cases, the HBV prevalence (20.7%) was significantly ($P < .01$) higher than that of nodal DLBCL (1.9%). By real-time PCP, the 2 examined splenic DLBCL cases had HBV DNA in their tumor tissues and lymphoma cells in 2 of 6 DLBCL cases showed an intranuclear reaction for the HBsAg. The findings support that HBV infection also influences the lymphomagenesis of B cells.

This is the first report regarding the significant prevalence of HCV and HBV infections in splenic DLBCL cases, which mainly show a favorable clinical course after splenectomy and cytotoxic treatments. Type II CG and rearrangement of the *Bcl2* gene in the tumor tissue were not found in the examined cases of HCV-positive splenic DLBCL. Further studies are necessary to identify the effects of HV infections, especially HCV, in splenic DLBCL cases.

References

[1] Ferri C, Caracciolo F, Zignego AL, et al. Hepatic C infection in patients with non-Hodgkin's lymphoma. Br J Haematol 1994;88: 392–4.

[2] Silvestri F, Pipan C, Barillari G, et al. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. Blood 1996;87:4296–301.

[3] De Vita S, Sacco C, Sansonno D, et al. Characterization of overt B-cell lymphomas in patients with hepatitis C virus infection. Blood 1997;90:776–82.

[4] Arcaini L, Paulli M, Boveri E, et al. Splenic and marginal zone lymphoma are indolent disorders at high hepatitis C virus seroprevalence with distinct presenting features but similar morphologic and phenotypic profiles. Cancer 2004;100:107–15.
[5] Hermine O, Lefrere F, Bronowicki JP, et al. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. N Engl J Med 2002;347:89–97.
[6] Mizorogi F, Hiramoto J, Nozato A, et al. Hepatitis C virus infection in patients with B-cell non-Hodgkin's lymphoma. Int Med 2000;39: 112–7.
[7] Satoh T, Yamada T, Nakano S, et al. The relationship between splenic malignant lymphoma and chronic liver disease associated with hepatitis C virus infection. Cancer 1997;80:1981–8.
[8] Lymphoma study group of Japanese pathologists. The World Health Organization classification of malignant lymphomas in Japan. Pathol Int 2000;50:696–702.
[9] Sansonno D, De Vita S, Cornacchiulo V, et al. Detection and distribution of hepatitis C virus–related proteins in lymph nodes of patients with type II mixed cryoglobulinemia and neoplastic or nonneoplastic lymphoproliferation. Blood 1996;88:4638–45.
[10] Locatelli GA, Spadari S, Maga G. Hepatitis C virus NS3 ATPase/ helicase. Biochemistry 2002;41:10332–42.
[11] Cocquerel L, Kuo CC, Dubuisson J, Levy S. CD81-dependent binding of hepatitis C virus E1 E2 heterodimers. J Virol 2003;77:10677–83.
[12] Zuckerman E, Suckerman T, Sahar D, et al. The effect of antiviral therapy on t(14;18) translocation and immunoglobulin gene rearrangement in patients with chronic hepatitis C virus infection. Blood 2001;97:1555–9.
[13] Galun E, Livni N, Ketzinei M, et al. Hepatitis B virus infection associated with hematopoietic tumors. Am J Pathol 1994;154:1001–7.
[14] Simmonds P, Holmes EC, Cha TA, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993;74: 2391–9.
[15] Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–42.
[16] Zanella I, Rossini A, Domenighini D, et al. Quantitative analysis of hepatitis B virus DNA by real time amplification. Eur J Clin Microbiol Infect Dis 2002;21:22–7.
[17] Jaffe ES, et al, editors. Tumors of haematopoietic and lymphoid tissues. WHO classification of tumors. Lyon: IARC Press; 2001, 121–187.
[18] Saadoun D, Suarez F, Lefrere F, et al. Splenic lymphoma with villous lymphocytes, associated with type II cryoglobulinemia and HCV infection. Blood 2005;105:74–6.
[19] Lunel F, Musset L, Cacoub P, et al. Cryoglobulinemia in chronic liver diseases. Gastroenterology 1994;106:1291–300.
[20] Ivanovski M, Silvestri F, Pozzato G, et al. Somatic hypermutation, clonal diversity, and preferential expression of the *VH* 51 p1/VL kv325 immunoglobulin gene combination in hepatitis C virus associated immunocytomas. Blood 1998;91:2433–42.
[21] Miklos JA, Swerdlow SH, Bahler DW. Salivary gland mucosa–associated lymphoid tissue lymphoma immunoglobulin *VH* genes show frequent use of V1-69 with distinct CDR3 features. Blood 2000;95:3878–84.
[22] Silvestri F, Barillari G, Fanin R, et al. Impact of hepatitis C virus infection on clinical features, quality of life and survival of patients with lymphoplasmacytic lymphoma/immunocytoma. Ann Oncol 1998;9:499–504.
[23] Takeshita M, Sakai H, Okamura S, et al. Prevalence of hepatitis C virus infection in cases of B-cell lymphoma in Japan: histological and etiologic differences of the southern European cases. Histopathology; in press.
[24] Tanaka K, Aiyama T, Imai J, et al. Serum cryoglobulinemia and chronic hepatitis C virus disease among Japanese patients. Am J Gastroenterol 1995;90:1847–52.
[25] Lenzi M, Frisoni M, Mantovani V, et al. Haplotype HLA-B8-DR3 confers susceptibility to hepatitis C virus–related mixed cryoglobulinemia. Blood 1998;91:2062–6.
[26] Nagasaka A, Takahashi T, Sasaki T, et al. Cryoglobulinemia in Japanese patients with chronic hepatitis C virus infection: host genetic and virological study. J Med Virol 2001;65:52–7.
[27] Murakami Y, Hotei H, Tsumura H, et al. A case of primary splenic malignant lymphoma and review of 98 cases reported in Japan. J Jpn Clin Surg 1988;49:716–9.
[28] Dammacco F, Sansonno D, Piccoli C, Tucci FA, Racanelli V. The cryoglobulinemias: an overview. Eur J Clin Invest 2001;31: 628–38.
[29] Bronowicki JP, Bineau C, Feugier P, et al. Primary lymphoma of the liver: clinicopathological features of relationship with HCV infection in French patients. Hepatology 2003;37:781–7.
[30] Luppi M, Ferrari MG, Bonaccorsi G, et al. Hepatitis C virus infection in subsets of neoplastic lymphoproliferations not associated with cryoglobulinemia. Leukemia 1996;10:351–5.
[31] Sakamuro D, Furukawa T, Takegami T. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69: 3893–6.
[32] Hofmann WP, Hermann E, Kronenberger B, et al. Association of HCV related mixed cryoglobulinemia with specific mutational pattern of the HCV E2 protein and CD81 expression on peripheral B lymphocytes. Blood 2004;104:1228–9.
[33] Szollosi J, Horejsi V, Bene L, et al. Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J Immunol 1996;157:2939–46.
[34] Ferrer M, Yunta M, Lazo PA. Pattern of expression of tetraspanin antigen genes in Burkitt lymphoma cell lines. Clin Exp Immunol 1998;113:346–52.
[35] Sasso EH, Martinez M, Yarfitz SL, et al. Frequent joining of *Bcl-2* to a *JH6* gene in hepatitis C virus–associated t(14;18). J Immunol 2004;143:3549–56.
[36] Boiocchi M. Low frequency of Bcl-2 rearrangement in HCV-associated non-Hodgkin's lymphoma tissues. Leukemia 2003;17: 1433–6.
[37] Machida K, Cheng KTN, Sung VMC, et al. Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. Proc Natl Acad Sci 2004;101:4262–7.
[38] Stoll-Becker S, Repp R, Glebe D, et al. Transcription of hepatitis B virus in peripheral blood monoclear cells from persistently infected patients. J Virol 1997;71:5399–407.
[39] Galun E, Ilan Y, Livni N, et al. Hepatitis B virus infection associated with hematopoietic tumors. Am J Pathol 1994;145:1001–7.
[40] Kim JH, Bang Y-J, Part BJ, et al. Hepatitis B virus infection and B-cell non-Hodgkin's lymphoma in a hepatitis B endemic area. A case control study. Jpn J Cancer Res 2002;93:471–7.

臨牀消化器内科 2005 Vol.20 No.5
©日本メディカルセンター

● 薬の知識

アデホビル

加藤　道夫*

はじめに

B型肝炎ウイルス(HBV)キャリアはHBe抗原陽性無症候性キャリアから慢性肝炎，肝硬変，肝細胞癌あるいは臨床的治癒とされているHBe抗体陽性無症候性キャリアまで，さまざまな病態が存在する．そして，その経過もさまざまであるが，大別すると肝硬変，肝細胞癌に進行する群と臨床的治癒の状態に落ち着く群に二分される．約80%は後者になると考えられるが，B型肝癌死亡者数も年間約5,000名を数え，これに肝不全死や劇症化による死亡を加えるとB型肝疾患による死亡者数は年間7,000～8,000名に上ると考えられている．

B型肝疾患の予後改善はHBe抗原が陰性化しHBV-DNA量低値が持続することによって得られ，対象例には適切な抗ウイルス治療がきわめて重要と考えられる．これまでB型慢性肝炎に対する抗ウイルス薬としてはインターフェロンとラミブジンの2薬のみが保険適用薬であったが，2004年12月より新たにアデホビルが保険適用となり，抗ウイルス治療の幅が広がってきた．

I．アデホビルピボキシル

アデホビルは，1986年に発見された核酸誘導体の抗ウイルス薬で，ウイルス遺伝子の複製にDNAポリメラーゼ/逆転写酵素を必要とするHBV，ヒト免疫不全ウイルス(HIV)などのレトロウイルス，ヘルペスウイルスなどに強い抗ウイルス活性を示した．しかし，バイオアベイラビリティが低かったため，ピボキシル基を導入したバイオアベイラビリティの高いアデホビルピボキシル(ヘプセラ® 錠)が合成された．

当初，HIV感染症治療薬，B型慢性肝炎治療薬として開発が開始された[1]が，HIV感染症治療薬としての開発は途中断念され，B型慢性肝炎治療薬として米国で2002年9月に承認された．2004年現在，39カ国で承認されている．

II．安全性

腎機能障害と乳酸アシドーシスおよび脂肪沈着による重度の肝腫大(脂肪肝)が重大な副作用

Key words：アデホビル，アデホビルピボキシル，ラミブジン，B型慢性肝炎，YMDD変異

Michio Kato
*独立行政法人国立病院機構大阪医療センター消化器科(〒540-0006 大阪市中央区法円坂2-1-14)

と考えられる．本剤投与中は腎機能障害の発現に注意し，とくに，腎機能障害のある患者やその既往歴のある患者においては，血清クレアチニンおよび血清リンの変動を定期的に観察する必要がある．国内第III相臨床試験(36例)における副作用としては悪心嘔吐，背部痛，NAG増加およびALP増加が各1例報告されている[2]．

III．臨床効果

アデホビルはYMDD野生株に対する有効性も報告されている[3]が，現在，本邦での保険適用はラミブジン投与中にHBVの持続的な再増殖を伴う肝機能の異常が確認されたB型慢性肝炎およびB型肝硬変例である．国内第III相臨床試験[2](16週投与)においてHBV-DNA変化量はラミブジンとの併用投与によって，16週後−3.75 log 10 copies/mlと著明に減少した(図1)．またALT正常化率も16週後に72%(26/36)と高率であった(図2)．耐性ウイルスに関しては少数報告されている[4),5]が，これらはラミブジンに対して感受性をもっているので，ラミブジンとの併用時には大きな障害にはならないと考えられる．

IV．症　例

アデホビル投与が有効であったHBe抗原陽性B型慢性肝炎の1例を呈示する(図3)．

HBステージ[6]IIb．ラミブジン投与前の肝組織診断は新犬山分類A2F3．投与直前のHBV-DNA量は3.5 Meq/ml，ALTは126 IU/lであった．

2000年7月11日よりラミブジン150 mg/dayを開始，2001年2月より100 mg/dayとし，現在まで続行中である．HBV-DNA量は速やかに低下し，2001年9月にPCR法で感度以下となった．その後，順調に経過していたが，2002年2月ごろより，HBV-DNA量の増加が持続し，2002年3月にYMDD変異株(YIDD)の出現を確認した．同時期よりALTの上昇とともに血清アルブミン値が低下し始め，同年11月には2.6 g/dlまで低下し，その状態が持続したため，2003年4月21日より個

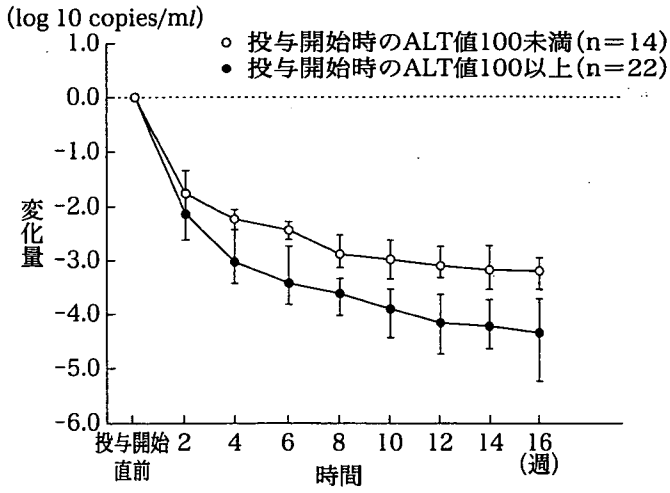

図1　アデホビル投与開始時のALT値別HBV-DNA変化量(国内第III相臨床試験)

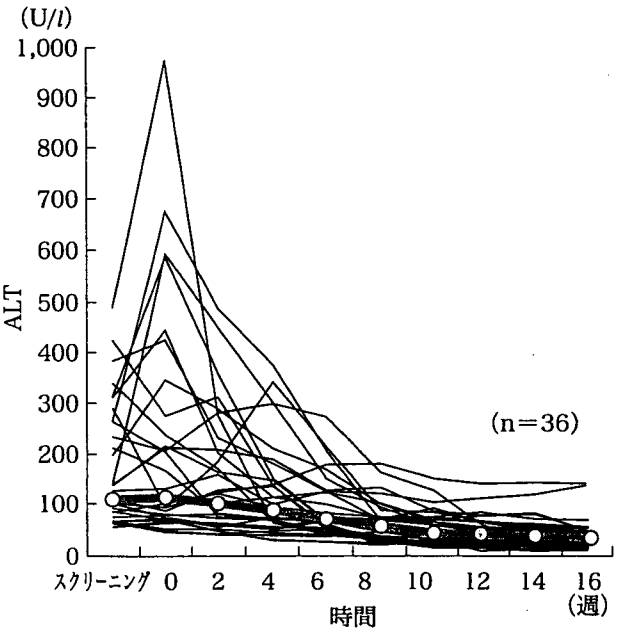

図2　アデホビル投与期間中のALT値の推移(国内第III相臨床試験)

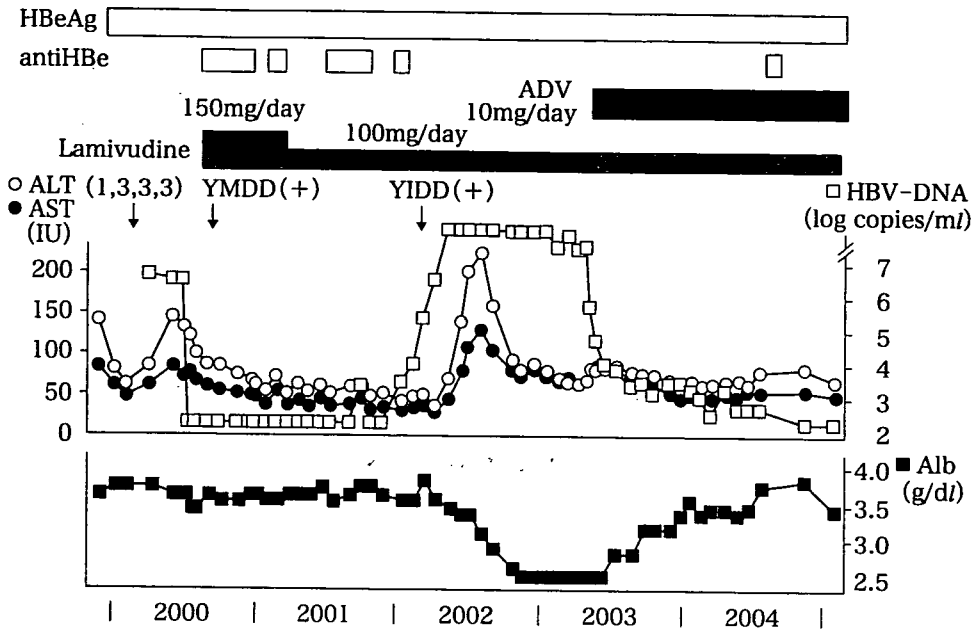

図3　アデホビル投与が有効であったYMDD変異株出現B型慢性肝炎例

人輸入にてアデホビル10 mg/dayの投与を開始した.

HBV-DNA量はアデホビル投与開始時7.2 log copies/m*l*であったが，投与1カ月後4.7，2カ月後3.9，6カ月後3.3と低下し，2004年9月より感度以下が持続している．血清アルブミン値もアデホビル投与開始時2.6 g/d*l*であったが，2カ月後2.9，6カ月後3.3と増加し，2004年10月には4.0 g/d*l*まで増加した．HBe抗原価もアデホビル投与開始後，漸次低下し，最終観察時点で3.9 C.O.Iとなり，HBe抗原の陰性化が期待できる状態である．

V．YMDD変異株出現とbreakthrough hepatitis

前述の症例ではYMDD変異株出現に伴って，いわゆるbreakthrough hepatitisが生じたが，HBV-DNA量の増加は認められるが，AST，ALTは正常値が持続し，breakthrough hepatitisを起こさない例やYMDD変異株が出現してもHBV-DNA量がほとんど増加しない例も存在する．breakthrough hepatitisが生じた群をA群，HBV-DNA量の増加は認められるがbreakthrough hepatitisを起こさない群をB群，YMDD変異株が出現してもHBV-DNA量がほとんど増加しない群をC群とすると，A群，B群ではHBVのRNA依存性DNAポリメラーゼのC-ドメインの変異(YVDD，YIDD)とともにB-ドメイン180番目のロイシンからメチオニンへの変異がきわめて高頻度で認められるが，C群ではC-ドメインの変異のみであることが多い．

A群とB群の判別は現在，明らかではないが，時期の問題か，生体側の因子が関係している可能性が考えられる．C-ドメインの変異(YVDD，YIDD)が認められたら，慎重に経過を観察し，breakthrough hepatitis増悪時には速やかなアデホビルの導入を考慮する必要がある．

おわりに

B型慢性肝炎でHBe抗原陽性持続例やHBe抗原は陰性化してもHBV-DNA量5.0 log copies/m*l*以上が持続する例は発癌リスクが高く[6]，抗ウイルス治療の導入が必要となる．ラミブジン治療においてはYMDD変異株の高頻度の出現が導入を逡巡させる一因であったが，アデホビルがB型慢性肝炎治療の選択肢に加わったことで，抗ウイルス治療の安全性と利便性が大きく広がったと考えられる．

文　献

1) Deeks, S. G., Collier, A., Lalezari, J., et al.：The safety and efficacy of adefovir dipivoxil, a novel anti-human immunodeficiency virus(HIV)therapy, in HIV-infected adults：a randomised, double blind, placebo-controlled trial. J. Infect. Dis. 176；1517-1523, 1997
2) 谷川久一，熊田博光，佐田通夫，他：YMDD変異ウイルスの増殖により肝機能の異常が認められたB型慢性肝炎患者(B型肝硬変患者を含む)に対するヘプセラ錠(アデホビルピボキシル)の臨床効果．肝胆膵　50；193-211, 2005
3) Marcellin, P., Chang, T. T., Lim, S. G., et al.：Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N. Engl. J. Med.　348；808-816, 2003
4) Angus, P., Vaughan, R., Xiong, S., et al.：Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology　125；292-297, 2003
5) Villeneuve, J. P., Durantel, D., Durantel, S., et al.：Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. J. Hepatl.　39；1085-1089, 2003
6) 加藤道夫，伊与田賢也，結城暢一，他：HBVマーカーと発癌リスクよりみたHBVキャリアのステージ分類—適切な抗ウィルス治療の選択に向けて．肝臓　45；581-588, 2004

特集

B型慢性肝炎治療の最前線 インターフェロン療法

Interferon therapy for chronic hepatitis B

加藤 道夫
KATO Michio

肝臓の臨床最前線

Key words: IFN治療, HBステージ分類, IFN・ラミブジン併用治療, ペグIFN治療, 長期予後

HBVキャリアはHBe抗原陽性無症候性キャリアから慢性肝炎，肝硬変，肝細胞癌あるいは臨床的治癒とされているHBe抗体陽性無症候性キャリアまでさまざまな病態が存在する．そして，その経過もさまざまであるが，大別すると肝硬変，肝細胞癌に進行する群と臨床的治癒の状態に落ち着く群に二分される．約80%は後者になると考えられるが，B型肝癌も全肝細胞癌中10～15%を占め，現在，死亡者数は横ばいで年間約5,000名を数えている．これに，肝不全死や劇症化による死亡を加えるとB型肝疾患による死亡者数は年間7,000名～8,000名に上ると考えられる．B型慢性肝炎の予後改善にはHBe抗原の陰性化とHBV-DNAの低値持続が必要であり，そのためには適切な抗ウィルス治療が肝要となる．現在，インターフェロン(IFN)，ラミブジンおよびアデホビルが保険適用製剤であるが，本稿ではB型慢性肝炎に対するIFN治療の現状と今後の展望について述べる．

Ⅰ．HBVキャリアのステージ分類とIFN治療対象の位置づけ

われわれはB型慢性肝炎の肝硬変進展，肝癌発癌抑止を目的とした適切な抗ウィルス治療の選択に向けての，HBVマーカーと発癌リスクよりみたHBVキャリアのステージ分類を提唱した[1)](表1)．

独立行政法人大阪医療センター消化器科　部長

HBステージ0

HBs抗原陽性，HBe抗原陽性，ALT正常値持続のいわゆる無症候性キャリアの状態．発癌リスクはほとんどなく，抗ウィルス治療の適応なし．

HBステージⅠ

HBs抗原陽性，HBe抗原陽性，ALT異常値(持続正常以外)でHBV-DNA量が$10^{7.6}$copies/mL以上の高ウィルス群．若年例(男性：30歳未満，女性：35歳未満)をステージⅠa，高年例(男性：30歳以上，女性：35歳以上)をステージⅠbとする．ステージ1a群も発癌リスクがきわめてまれで，

0371-1900/05/¥50/頁/JCLS

表1 HBV キャリアのステージ分類

HB stage	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ
HBsAg	＋	＋	＋	＋	＋	**－
HBeAg	＋	＋	＋	－	－	－
HBV-DNA (copies/ml)	不問	$10^{7.6}$≦	$10^{7.6}$＞	10^{5}≦	10^{5}＞	不問
ALT	持続正常	*持続正常以外	*持続正常以外	不問	不問	不問
年齢	不問	若年/高年 (Ⅰa/Ⅰb)	若年/高年 (Ⅱa/Ⅱb)	不問	不問	不問
発癌リスク	きわめて小	小/大	小/きわめて大	きわめて大	きわめて小	きわめて小

*若年：男性30歳未満，女性35歳未満　** HBsAg(＋)の時期が確認されていること
高年：男性30歳以上，女性35歳以上

通常は抗ウィルス治療の必要はないが組織学的に線維化ステージが進行している例は抗ウィルス治療の適応となる．ステージⅠa，Ⅱaとも薬剤としては若年で免疫応答が良好であるのでIFNが第一選択となると考える．IFNについてわれわれは後述の少量間歇投与が若年例に有効であることを報告[2]したが，特に30歳未満例にはIFN治療は長期投与でなくても有効性は高いと考える．一方，ステージⅠb群は発癌リスクを有し，抗ウィルス治療の必要を認める．HBV-DNA量がきわめて高値のこの群はラミブジン単独での治療効果の持続は困難で，エンテカビル等の抗ウィルス効果の強い薬剤あるいはIFN/ラミブジン併用治療が適応になると考えられる．

HB ステージⅡ

HBs抗原陽性，HBe抗原陽性，ALT異常値(持続正常以外)でHBV-DNA量が$10^{7.6}$copies/mL未満の低ウィルス群．若年例をステージⅡa，高年例をステージⅡbとする．ステージⅡa群は発癌リスクは少ないが若年発癌例が存在し，またALT高値が持続する例も多く，抗ウィルス治療の適応になる．ステージⅡb群は発癌リスクがきわめて大で抗ウィルス治療の絶対適応である．ラミブジン等の核酸アナログ単独あるいはIFN，HBワクチンとの併用の選択が考えられる．

HB ステージⅢ

HBs抗原陽性，HBe抗原陰性，HBV-DNA 10^{5}copies/mL以上のpre-core mutant株のreplicationが持続している群である．発癌リスクはきわめて大で，ALT値異常のとくに男性はステージⅡbとともに抗ウィルス治療の絶対適応である．薬剤としては高年例が大半を占め，ラミブジンの治療効果が良好でYMDD変異株の出現も低率であるため，現在のところラミブジンが第一選択であり，YMDD変異株出現例にはアデホビル等の他の核酸アナログの併用あるいは切り替えで対応できると考えられる．

HB ステージⅣ

HBs抗原陽性，HBe抗原陰性，HBV-DNA 10^{5}copies/mL未満のいわゆる臨床的治癒の状態である．発癌リスクとしてはきわめてまれで原則的には抗ウィルス治療の必要はないと考える．

HB ステージⅤ

HBキャリア(HBs抗原陽性の時期が確認されている例)でHBs抗原が消失した状態である．HBステージⅣと同様，発癌リスクはきわめてまれで抗ウィルス治療の必要はない．

HBVキャリアの大多数が歩む臨床的治癒状態へのコースはステージⅠaからステージⅡaとなり，その後短期間ステージⅢを経由した後速やかにステージⅣに移行するものと考えられる．そしてステージⅣが長期間続いた後HBs抗原が消失し，ステージⅤとなる．一方，肝硬変進展・肝癌発癌ハイリスク群はステージⅠaからステージ

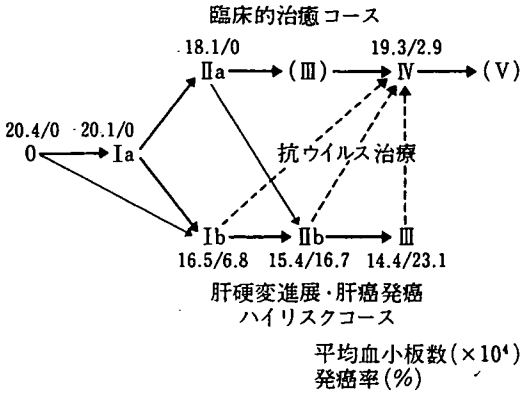

図1　HBV キャリアの経過(臨床的治癒コースと肝硬変進展・肝癌発癌ハイリスクコース)

Ⅰb，ステージⅡb と進行し，HBe 抗原が陰性化してステージⅢまでは到達するが HBV の増殖は持続し，ステージⅣに至ることはない(図1)．臨床的治癒コースの各ステージにおける初診時の血小板数と発癌リスクは，ステージ0，Ⅰa，Ⅱa およびⅣでそれぞれ20.4万/0%，20.1万/0%，18.1万/0%および19.3万/2.9%とほとんど変化を認めないが，肝硬変進展・肝癌発癌ハイリスクコースにあたるステージⅠb，Ⅱb およびⅢではそれぞれ16.5万/6.8%，15.4万/16.7%および14.4万/23.1%とステージの移行にしたがっての血小板数の低下と発癌率の増加が認められ，ステージⅠb，Ⅱb およびⅢのキャリアに対する抗ウィルス治療の必要性が強く示唆される．特に IFN 治療の適用対象としては，HBe 抗原陽性若年例(ステージⅠa，Ⅱa)では IFN 単独，HBe 抗原陽性高年例(ステージⅠb，Ⅱb)では IFN/ラミブジン併用治療(後述)が適切な選択ではないかと考える．

Ⅱ．これまでの IFN 治療

1．少量間欠投与

われわれは1984年より HBe 抗原陽性例に対して natural IFNα の少量間欠投与を行い，良好な成績を報告[2]した．投与法は大阪府赤十字血液センターより供与をうけたヒト白血球 IFN を週1回計4回(初回量2.4MU～3MU，以下漸減投与)総量6.8MU～10MU の投与である．投与対象の性別は男性9例，女性6例で，それぞれの年齢は男性20歳～55歳(平均35.8歳)，女性22歳～45歳(平均31.7歳)であった．成績は投与終了後6ヵ月での HBe 抗原陰性化率53.3%(図2)，HBe 抗原抗体 seroconversion 率33.3%および ALT 正常化率53.3%といずれも高率であった．HBe 抗原陰性化例の多くは投与終了後，ALT の上昇後に消失し，女性，HBe 抗原価低値，投与前 ALT 高値および組織診断で activity の高い症例に得られやすいことが判った．対象の60%が35歳未満の若年であったことが良好な成績が得られた要因と考えられ，HB ステージⅠa，Ⅱa で抗ウィルス治療

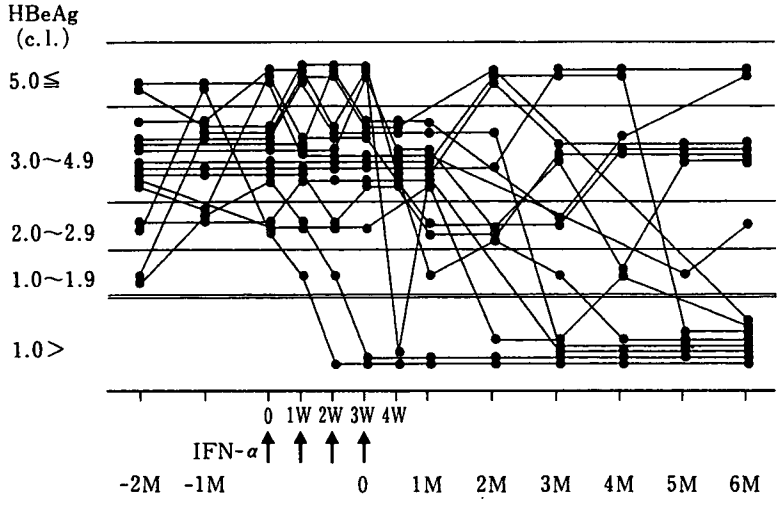

図2　IFN 少量間欠投与前後の HBe 抗原カットオフインデックスの変動

表2 B型慢性肝炎に対するIFN4週投与と24週投与の比較検討

	投与終了時		投与終了6ヵ月後	
ALT正常化				
S群	2/26(7%)	*	3/23(13%)	
L群	10/25(40%)		8/25(32%)	
H群	20/25(80%)	**	9/25(36%)	
HBV-DNA陰性化				
S群	5/26(19%)		3/23(13%)	
L群	6/23(26%)		5/20(25%)	
H群	11/22(50%)		8/23(35%)	
Seronegative				
S群	2/25(8%)		1/25(4%)	
L群	4/25(16%)		6/22(27%)	***
H群	7/25(28%)		6/24(25%)	
L+H群	11/50(22%)		12/46(26%)	
Seroconversion				
S群	2/25(8%)		1/25(4%)	
L群	4/25(16%)		4/22(18%)	
H群	6/25(24%)		5/24(21%)	

*p <0.02, **p <0.0001, ***p <0.05

の必要な症例には有用な治療法と考えている.

2. 4週連日投与

西口[3]はわが国のIFN 4週投与の成績をまとめ，投与終了1年後，2年後のHBe抗原陰性化率はそれぞれ29%，55%，HBe抗原抗体seroconversion率は12%，29%で自然経過よりも高率であるとしている．われわれ[4]もHBe抗原陽性例23例(男性16例，女性7例，平均年齢36.3歳)に対してIFNα-2a 9MU 3日間連日投与後，18MU 25日間連日投与の計477MUの投与を行った．成績は投与終了1年後のHBe抗原陰性化率50.0%，ALT正常化率36.8%およびHBV-DNA陰性化率41.2%であった．4週投与においても1回投与量高用量の製剤を用いることによって，6ヵ月投与と同等の総投与量を投与でき，また，対象に若年例が多かったことが，良好な成績が得られた要因と考えている.

3. 長期24週投与

2000年4月より24週の長期投与が可能となった．24週投与の最大の利点は投与期間中にHBe抗原抗体seroconversionが生じる可能性が高く，投与終了後の急性増悪の出現を防止できることである．4週投与との比較として大阪大学旧第1内科関連病院肝臓グループで行ったIFNの長期投与試験の成績[5]を紹介する．IFN投与スケジュールはS群600万単位4週間連日投与，L群300万単位4週間連日投与後，週3回20週投与，H群600万単位4週間連日投与後，週3回20週投与の3群間の比較試験である．治療成績は表2に示すようにALTの改善率，HBV-DNAの消失率とも4週投与(S群)に比較して24週投与(L群，H群)の方が，投与直後および投与6ヵ月とも高率であった．また，HBe抗原陰性化率，HBe抗原抗体seroconversion率でも，4週投与(S群)に比較して24週投与(L群，H群)の方が高率であり，24週の長期投与の有効性が高いと考えられた．西口の国内治療成績の集計でも，投与終了6ヵ月後のHBe抗原陰性化率は4週投与，24週投与でそれぞれ11%，28%と長期投与の有効性が確認されている．欧米では6ヵ月投与が標準投与法であるが，Wongら[6]の比較対照試験の集計でも投与終了後6ヵ月の時点でのHBe抗原陰性化率33%と，自然経過例12%に比し有意に高率であったとしている.

Ⅲ. これからのIFN治療

1. IFN・ラミブジン併用治療

HBe抗原陽性例に対するIFN治療は24週の長期投与でも，満足できる成績は得られていない．また，ラミブジン治療においてもHBe抗原陽性のHBV-DNA高値例ではHBe抗原陰性化率は低率である．われわれの施設ではHBe抗原陽性例に対するIFN・ラミブジン併用治療とIFN単独治療の比較検討を行っている．併用治療のプロトコールはIFNα6MU2週連投後22週週3回投与とラミブジン100mg連日投与を同時に投与開始し，24週後よりはラミブジン単独投与を継続するものである．現在，治療成績を解析中であるが，併用群においてMeriganのType Ⅰ効果にあたるHBs抗原陰性化例を2例(genotype AおよびC)認めている．HBステージⅠb，Ⅱbはともに HBe抗原陽性期であるが，われわれはこの時期においても大半の例はpre C mutantの出現が確認され，また，IFNはpre C wild，pre C mutantのいずれの株にも同等に有効であることを報告[4]した．ラミブジンはpre C mutant株に対してより強い抗ウィルス効果が得られる[7]こと，また，ラミブジンのYMDD変異株はIFN前投与あるいは併用群において出現率が低率であること(自験例，未発表)を考慮すると，ステージⅠb，Ⅱb群にはラミブジン単独よりもIFNとの併用がより有効ではないかと考えている．Schalmら[8]，Serfatyら[9]もIFN/ラミブジン併用治療の有効性について報告している．

2. ペグIFN治療

C型慢性肝炎には国内で2003年12月より週1回投与のペグIFNα-2a治療が保険適用になったが，B型慢性肝炎に対しても海外ではペグIFNα-2aを用いたトライアルが行われている．まず，Cooksleyら[10]はHBe抗原陽性例に対するphase Ⅱ studyを実施し，従来のIFNα-2a製剤に比しペグIFNα-2a製剤は有意に有効性が高いと報告している．また，Marcellinら[11]はHBe抗原陰性例に対してペグIFNα-2a単独，ペグIFNα-2a/ラミブジン併用およびラミブジン単独の比較試験を行い，ペグIFNα-2a単独群およびペグIFNα-2a/ラミブジン併用群はラミブジン単独群に比し，投与終了後6ヵ月でのHBV-DNA陰性化率，ALT正常化率はともに高率であったと報告している．

3. HBe抗原陰性例に対するIFN治療

HBe抗原陰性例に対するIFN治療の有用性に関しては一定の見解が得られていないが，積極的に使用を勧める報告[12][13]は少なく，わが国では現在保険適用になっていない．HBe抗原陰性例はHBステージのⅢとⅣにあたるが，臨床的治癒の状態であるステージⅣは治療の必要はなく，pre C mutantの増殖が持続するステージⅢが抗ウィルス治療の対象となる．ステージⅢの平均年齢は53歳[1]で，大半の症例が40歳以上である．ラミブジン治療ではHBe抗原陰性例の方がYMDD変異株出現率が低く治療効果も良好で，アデホビルも使用可能となったことから，HBe抗原陰性例に対してはラミブジン治療が第一選択と考えられる．IFN治療としてはペグIFN長期投与に期待したいところである．

Ⅳ. IFN治療例の長期予後と肝発癌

最後にIFN治療例の長期予後について述べる．1981年8月より1992年12月までにIFN治療を開始したB型慢性肝炎102例(平均観察期間7.3年)を対象に長期予後と肝癌発癌について検討した[14]．102例の性別は男性64例，女性38例，平均年齢はそれぞれ34.3歳，35.7歳で，使用したIFNの総投与量は6.8MU～1284MU(平均143.6MU)であった．投与終了後12～15年後(平均年齢約50歳)の累積HBe抗原陰性化率は90%，累積ALT正常化率は80%と80%の症例は臨床的治癒の状態とな

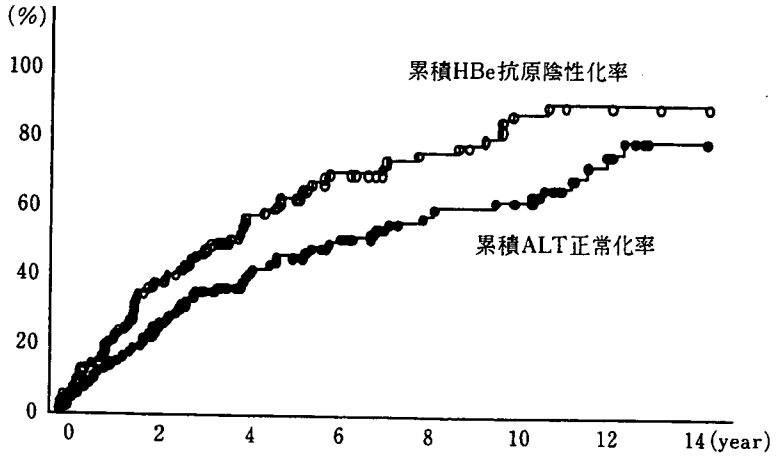

図3 累積HBe抗原陰性化率と累積ALT正常化率(カプラン・マイヤー法)

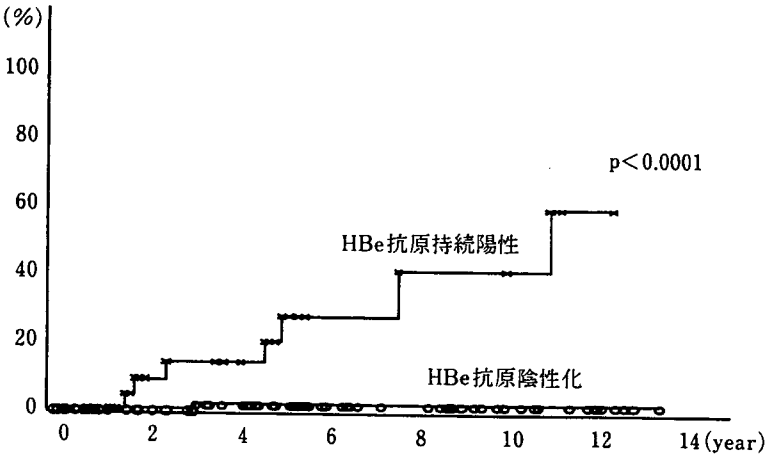

図4 累積HCC発癌率(カプラン・マイヤー法) HBe抗原陰性化の有無による検討

るが，20%はALTの異常が持続することが判った(図3)．ALT異常が持続する半数はHBe抗原持続陽性例でHBステージⅠb，Ⅱbに相当し，残りの半数はHBe抗原陰性でpre C mutantの増殖が持続するステージⅢと考えられる．肝癌発癌は8例(全例男性)に認められた(年率1.08%)．そのうちHBe抗原持続陽性例は7例(年率4.1%)，HBe抗原陰性化例は1例(年率0.29%)でHBe抗原持続陽性例の発癌リスクはHBe抗原陰性化例の約14倍であった．累積肝癌発癌率の検討でもHBe抗原持続陽性例で有意に高率であった(図4)．HBe抗原陰性化は，年齢が若年であるほど，壊死，炎症が高度なほど有意に高率であり，若年齢で組織学的に壊死，炎症の強い時期にIFN治療を開始することがHBe抗原陰性化さらに発癌抑止につながると考えられた．

おわりに

これまでIFNとラミブジンのみが保険適用の抗ウィルス剤であったが，本年12月よりアデホビルが保険適用となり，B型慢性肝炎に対する抗ウィルス治療も新しい局面を迎えた．B型はC型に比し治療対象の選択がより重要で，それぞれの対象に対する適切な治療方法の選択と的確な治療の遂行が肝不全や発癌を防止し，予後の改善に寄与すると考えられる．今後，B型慢性肝炎に対しても早期のIFN自己注射とペグIFNの使用が望まれるところである．

文　献

1) 加藤道夫，伊与田賢也，結城暢一，ほか：HBV マーカーと発癌リスクよりみたHBV キャリアのステージ分類－切な抗ウィルス治療の選択に向けて－．肝臓　45：581-588，2004.
2) 加藤道夫，益沢　学，奥山卓正，ほか：B型慢性肝炎に対するヒト白血球インターフェロン少量間歇投与．肝臓　27：552-560，1986.
3) 西口修平：IFN 治療．コンセンサス肝疾患2002－診断・治療と病態"B型肝炎治療"，矢野右人監修，p71-77，日本メディカルセンター，東京，2002.
4) Kato M, Yuki N, Kaneko A, et al：Changes in virus loads and precore mutations in chronic hepatitis B patients treated with 4 weeks of daily interferon alfa-2a therapy. Hepatol Res 28：73-78, 2004.
5) 林　紀夫，加藤道夫：B型慢性肝炎に対するIFN 治療の長期予後とIFN の長期投与．第22回犬山シンポジウム，B型肝炎の新しい展開．犬山シンポジウム記録刊行会編，p68-69，中外医学社，東京，2001.
6) Wong DK, Cheung AM, O'Rourke K, et al：Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B：A meta-analysis. Ann Intern Med 119：312-323, 1993.
7) Cho SW, Hahn KB, Kim JH, et al：Reversion from precore/core promoter mutants to wild type hepatitis B virus during the course of lamivudine therapy. Hepatology 32：1163-1169, 2000.
8) Schalm SW, Heathcote J, Cianciara J, et al：Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection：a randomized trial. Gut 46：562-568, 2000.
9) Serfaty L, Thabut D, Zoulim F, et al：Sequential treatment with lamivudine and interferon monotherapies in patients with chronic hepatitis B not responding to interferon alone. Hepatology 34：573-577, 2001.
10) Cooksley WGE, Piratvisuth T, Lee S-D, et al：Peginterferon alfa-2a (40kDa)：an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. J Viral Hepatitis 10：298-305, 2003.
11) Marcellin P, Lau G, Bonino F, et al：Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patientswith HBeAg-negative chronic hepatitis B.N Engl J Med 351：1206-1217, 2004.
12) Kako M, Kanai K, Aikawa T, et al：Response to interferon-alpha 2a in patients with e antigen-negative chronic hepatitis B.J Clin Gastroenterol 25：440-445, 1997.
13) Lampertico P, Ninno ED, Vigano M, et al：Long-term suppression of hepatitis B e antigen-negative chronic hepatitis B by 24-month interferon therapy. Hepatology 37：756-763, 2003.
14) 加藤道夫，益澤　学：インターフェロン治療を行ったB型慢性肝炎の長期予後について．肝臓　39：679-681，1998.

ELSEVIER

Journal of Hepatology 44 (2006) 302–309

Journal of Hepatology

www.elsevier.com/locate/jhep

Significance of liver negative-strand HCV RNA quantitation in chronic hepatitis C

Nobukazu Yuki[1,*], Shinji Matsumoto[2], Kenichi Tadokoro[2], Kiyoshi Mochizuki[3], Michio Kato[1], Toshikazu Yamaguchi[2]

[1]*Department of Gastroenterology, Osaka National Hospital, Hoenzaka 2-1-14, Chuo-ku, Osaka 540-0006, Japan*
[2]*BML, Inc., Kawagoe 350-1101, Japan*
[3]*Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita 565-0871, Japan*

***Background/Aims*: Liver negative-strand hepatitis C virus (HCV) RNA is the most direct indicator of active viral replication but has only been examined in a few semiquantitative studies.**

***Methods*: Positive- and negative-strand HCV RNA in the right (R) and left (L) liver lobes was quantified by rTth-based strand-specific real-time polymerase chain reaction for 48 chronic hepatitis C patients.**

***Results*: Close correlations between lobes were seen for positive- and negative-strand amounts ($r=0.950$; $P<0.001$ and $r=0.920$; $P<0.001$, respectively). The ratio of negative to positive strands (median, 0.14 for R and 0.13 for L) varied by 2 log directly in relation to HCV replication assessed by liver negative strands but had no relation to liver positive strands and circulating HCV. Only negative-strand quantitation was inversely correlated with age ($r=-0.322$; $P=0.026$ for R and $r=-0.340$; $P=0.018$ for L), while liver tissues with hepatitis B virus DNA contained larger amounts of each strand. In 27 patients treated with enhanced interferon monotherapy, the amounts of liver negative strands (<4 log copies/100 ng RNA) were the only independent predictor of a sustained virologic response.**

***Conclusions*: Negative-strand quantitation is uniform in the liver and bears distinct relevance to the disease.**

***Keywords*: Negative-strand HCV RNA; HCV replication; Chronic hepatitis C**

1. Introduction

Hepatitis C virus (HCV) replication, like that of other single-strand, positive-sense RNA viruses, is presumably preceded by the synthesis of negative-strand RNA. Thus, the amounts of negative-strand RNA-replicative intermediates in liver tissues should serve as a more reliable marker of active viral replication than positive-strand HCV RNA in the liver or in circulation. Serum HCV loads are affected by replication within the liver and extrahepatic sites and by immunologic clearance of the virus. The detection of liver positive-strand (genomic) HCV RNA can simply imply contamination by such circulating virions. Thus far, only a few semiquantitative studies have been done on the clinical relevance of liver negative-strand HCV [1–4], and controversy remains. Patients with chronic hepatitis C can show uneven distribution of liver injury, but intrahepatic variation of HCV replication also remains to be clarified. To further address these issues, we quantitatively analyzed positive- and negative-strand HCV RNA in each liver lobe by strand-specific real-time polymerase chain reaction (PCR) using rTth.

Received 3 May 2005; received in revised form 22 October 2005; accepted 25 October 2005; available online 15 November 2005
* Corresponding author. Tel.: +81 6 6942 1331; fax: +81 6 6943 6467.
E-mail address: yuki@onh.go.jp (N. Yuki).

0168-8278/$30.00
doi:10.1016/j.jhep.2005.10.014

2. Patients and methods

2.1. Patients

Forty-eight patients with chronic hepatitis C underwent laparoscopic liver biopsies. All patients were positive for serum HCV RNA (Amplicor HCV Test, Roche Diagnostics K.K., Tokyo, Japan). No confounding etiology of liver disease was found in any patient. They were negative for hepatitis B surface antigen in the serum. The group was comprised of 25 men and 23 women ranging in age from 33 to 70 years (median, 57 years). Sixteen (33%) patients had a history of blood transfusion 8–52 years (median, 36 years) earlier. Biopsies were performed using 13-gauge Tru-Cut needles (Hakko Medical Co., Ltd, Nagano, Japan), and liver tissues sufficient for histologic and virologic evaluation were obtained from the anterior segment of the right lobe and the lateral segment of the left lobe. Specimens 15 mm long and 2 mm wide were embedded in paraffin for histopathological study. The remaining portions were immediately frozen and then stored at −80 °C until PCR testing. With one patient, the specimen from the left lobe was subjected to only virologic evaluation due to its limited size. Paired serum samples were obtained from all patients at laparoscopy and stored at −80 °C without thawing until virologic tests. Of the 48 patients, 27 (Table 1) were treated with enhanced interferon (IFN) monotherapy. After laparoscopy, 3 MU of IFN-β (Feron, Toray Co., Tokyo, Japan) was administered twice a day for 2 weeks followed by 9 MU of IFN-α (Sumiferon, Sumitomo Pharm. Co., Osaka, Japan) daily for 2 weeks and thrice weekly for 20 weeks. The study was approved by the local research ethics committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

2.2. Virologic testing

Circulating HCV genomic RNA was quantified by a PCR assay (Amplicor HCV Monitor Test version 2.0, Roche Diagnostics K.K.). HCV RNA of ≧6.4 log copies/mL was measured after serum dilution. HCV genotypes were determined by a PCR genotyping system [5].

2.3. Positive- and negative-strand HCV RNA quantitation by rTth-based strand-specific real-time reverse-transcription polymerase chain reaction (RT-PCR)

Strand-specific TaqMan RT-PCR was designed to quantify the 5′ untranslated region of the HCV genome using a thermostable enzyme, rTth (Applied Biosystems, Foster City, CA). Total hepatic RNA, 100 ng, was added to an RT reaction mixture containing 2 μL of 10× RT buffer (Applied Biosystems), 20 nmol of $MnCl_2$, 5 U of rTth, 24 U of RNasin (Promega, Madison, WI), 4 nmol of each dNTP, and 10 pmol of sense primer HCV-20F (5′-CGACACTCCACCATGAATCACT-3′) for the negative-strand assay or antisense primer HCV-114R (5′-GAGGCTG-CACGACACTCATACT-3′) for the positive-strand assay. The RT reaction was performed in a final volume of 20 μL at 70 °C for 60 min. The reaction

Table 1
Baseline patient characteristics before IFN therapy

		n
Age	<50	11 (41%)
	≥50	16 (59%)
Sex	Male	16 (59%)
	Female	11 (41%)
Transfusion history	+	9 (33%)
	−	18 (67%)
ALT	<2×ULN	17 (63%)
	≥2×ULN	10 (37%)
Liver histology		
Grading score[a]	<7	17 (63%)
	≥7	10 (37%)
Staging score[a]	<4	19 (70%)
	≥4	8 (30%)
Between-lobe grade discrepancy	+	10 (38%)
	−	16 (62%)
Between-lobe stage discrepancy	+	9 (35%)
	−	17 (65%)
HCV genotype	1	21 (78%)
	2	6 (22%)
Serum HCV RNA	<5.4 log copy/mL	6 (22%)
	≥5.4 log copy/mL	21 (78%)
+Strand[a]	<5 log copy/100 ng RNA	6 (22%)
	≥5 log copy/100 ng RNA	21 (78%)
−Strand[a]	<4 log copy/100 ng RNA	9 (33%)
	≥4 log copy/100 ng RNA	18 (67%)
−/+ Strand ratio[a]	<0.1	14 (52%)
	≥0.1	13 (48%)
Between-lobe + strand discrepancy	+	4 (15%)
	−	23 (85%)
HBV antibody (anti-HBc and/or anti-HBs)	+	7 (26%)
	−	20 (74%)
Liver HBV DNA	+	2 (7%)
	−	25 (93%)

[a] Mean values of right and left liver lobes.

was then treated with 5 μL of 10× chelating buffer (Applied Biosystems), 75 nmol of $MgCl_2$, 10 nmol of each dNTP, 10 pmol of antisense primer HCV-114R for the negative-strand assay or sense primer HCV-20F for the positive-strand assay, and 5 pmol of TaqMan probe HCV-P43 (5′FAM-CCCTGTGAGGAACTACTGTCTTCAC-GCAGATAMRA3′). The final volume was adjusted to 50 μL. The samples were promptly set in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and incubated at 70 °C for 2 min and then at 94 °C for 2 min. Real-time PCR amplification and data analysis were subsequently performed for 45 cycles (94 °C for 20 s and 62 °C for 1 min). Copy numbers of the 95-base target sequence were determined using the standard curve based on measurements of serial 10-fold dilutions of synthetic positive- and negative-strand HCV RNA. The sensitivity was 2 log copies/reaction for the positive-strand assay and 3 log copies/reaction for the negative-strand assay. The dynamic ranges were 2–7 log copies/reaction and 3–7 log copies/reaction, respectively. In each assay, false detection of an incorrect strand occurred when the amount of incorrect strand added reached 7 log copies. The positive- and negative-strand quantitation before normalization was ≦6.4 and ≦5.9 log copies/100 ng liver RNA, respectively, in this study. Thus, the strand-specificity was unlikely to be affected by an excess of incorrect strands. Self-priming or endogenous priming was ruled out by the lack of amplification product following RT-PCR of total hepatic RNA without primers in the RT mixture. All assays were done in duplicate, and the mean values were obtained. Hepatic RNA samples from the same liver were always measured in the same run.

The HCV-specific primers and probe used are conserved among genotypes. To verify that HCV genotypes 1b, 2a and 2b could be quantified with similar efficiency, high-concentration serum samples of each genotype were obtained from eight patients and diluted to 4.4 log copies/reaction by Amplicor HCV Monitor version 2.0, which is known to equally amplify all genotypes. The positive-strand HCV quantitation by the TaqMan RT-PCR was the same for genotypes 1b (5.3±0.7), 2a (5.5±0.4) and 2b (4.9±0.5 log copies/reaction) ($P=0.141$ by one-way analysis of variance).

2.4. Normalization of hepatic HCV RNA amounts and criteria for between-lobe discrepancies

GAPDH mRNA in total hepatic RNA, 100 ng, and control total RNA (Raji cell line), 100 ng, was also quantified by real-time RT-PCR, and copy numbers were determined using the standard curve (Human GAPD Endogenous Control, Applied Biosystems). Hepatic HCV RNA and GAPDH mRNA quantitation, which were performed in separate tubes, showed a linear relationship with the amounts of target RNA (Fig. 1). The HCV RNA copy number was divided by the ratio of the sample GAPDH mRNA amounts to the TaqMan control value. Thus, normalized hepatic HCV RNA amounts were obtained and used for data analysis. In preliminary experiments, assay variance for the $\log_{10}$ transformed HCV RNA quantitation before normalization was evaluated based on five measurements of 10 liver samples (intra-assay coefficients of variation (CVs)=0.88–2.85% and inter-assay CVs=1.19–6.91% for the positive-strand assay; intra-assay CVs=2.27–9.72% and inter-assay CVs=1.52–18.11% for the negative-strand assay). Assay variance was greater for the negative-strand assay, which may be attributable to interfering factor(s) such as a large amount of positive strands in the RT reaction. The mean SDs of intra-assay variance were 0.106 and 0.081 for <5 and ≧5 log copies, respectively, in the positive-strand assay, whereas they were 0.374, 0.256 and 0.158 for <4, 4–5 and ≧5 log copies, respectively, in the negative-strand assay. The HCV RNA quantitation was assumed to vary within twice these SDs. Between-lobe HCV RNA differences were considered significant when the normalized HCV RNA amounts differed by more than the estimated variance for normalized values. All discrepancies were confirmed by repeating the assays.

2.5. Detection of liver hepatitis B virus (HBV) DNA by nested PCR

Total hepatic DNA, 100 ng, was subjected to nested PCR to amplify HBV DNA. The primers were set in the surface region (outer sense 5′-TCGTGTTACAGGCGGGGTTT-3′; outer antisense 5′-CGAACCACT-GAACAAATGGC-3′; inner sense 5′-CAAGGTATGTTGCCCGTTTG-3′; inner antisense 5′-GGCACTAGTAAACTGAGCCA-3′) and the X region (outer sense 5′-GCATGGAGACCACCGTGAA-3′; outer antisense 5′-CAGACCAATTTATGCCTACAG-3′; inner sense 5′-TACATAAGAG-GACTCTTGGACT-3′; inner antisense 5′-CAGACCAATTTATGCCTA-CAG-3′). PCR products (233 and 151 bp, respectively) were visualized by 3% agarose electrophoresis and ethidium bromide staining. All assays were done in duplicate. The sensitivity was 1 copy/100 ng liver DNA for each primer set. To avoid contamination in all PCR assays, the contamination avoidance measures [6] were strictly applied throughout the study, and positive and negative controls were used.

2.6. Histologic evaluation

After routine staining with hematoxylin–eosin, all liver biopsy specimens were examined by the same experienced pathologist without knowledge of their source. Biopsy specimens were semiquantitatively

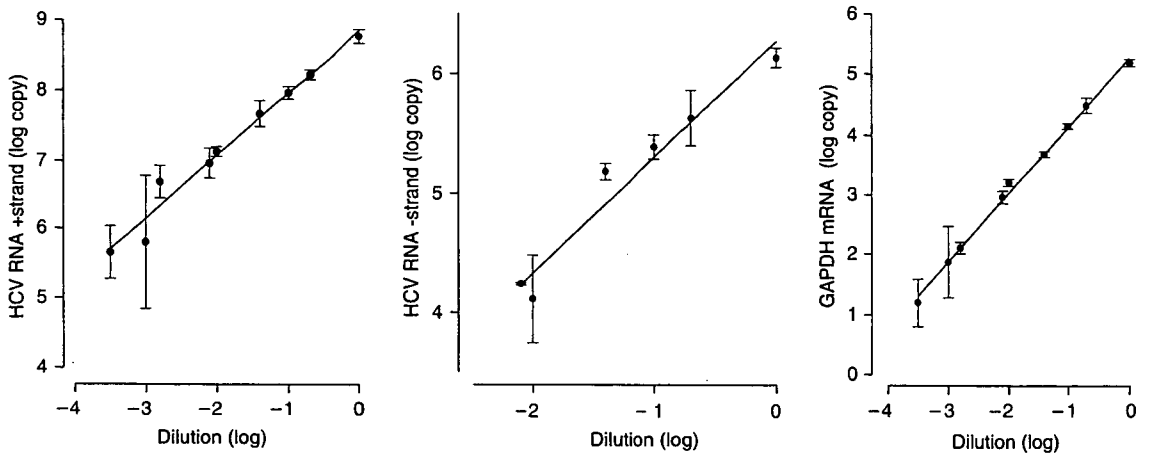

Fig. 1. Changes in hepatic HCV RNA and GAPDH mRNA quantitation in separate tubes according to the amounts of target RNA. Serial dilutions of total hepatic RNA, 100 ng, were subjected to real-time RT-PCR, and copy numbers were determined using the standard curve. The copy number (log) showed a linear relationship ($P<0.001$) with the amounts of target RNA expressed as dilution (log) ($y=8.86+0.90x$ [$n=9$, $r=0.985$] for positive-strand HCV RNA, $y=6.27+0.96x$ [$n=6$, $r=0.976$] for negative-strand HCV RNA and $y=5.27+1.13x$ [$n=9$, $r=0.998$] for GAPDH mRNA). Data are the mean±2SD of triplicate measurements. Pearson's correlation test was performed to examine the relationship.

evaluated using the modified histologic activity index described by Ishak et al. [7].

2.7. Statistical analysis

Viral load was $\log_{10}$ transformed to obtain a more symmetrical distribution without outliers. An arbitrary value of 0 log copy/100 ng liver RNA was attributed to the liver tissues negative by PCR. Data on continuous variables were presented as mean ± SD unless otherwise stated. Statistical analysis for group comparisons was performed using the Wilcoxon nonparametric test. Correlations between the variables were calculated using Spearman rank order correlations. To assess variables potentially related to virologic and histologic between-lobe discrepancies and responses to IFN, stepwise multivariate logistic regression models were used. All analyses were done with SAS (version 8.02) (SAS Institute, Inc., Cary, NC). A P value of less than 0.05 (2-tailed) was considered to indicate significance.

3. Results

3.1. Amounts of positive- and negative-strand HCV RNA in right (R) and left (L) liver lobes

Normalized positive-strand HCV loads in the right liver lobe (median, 5.9; range, 2.5–8.5 log copies/100 ng liver RNA) showed a correlation with those in the left liver lobe (median, 6.0; range, negative to 6.8 log copies/100 ng liver RNA) ($r=0.950$; $P<0.001$) (Fig. 2A). Six (13%) of the 48 patients had a between-lobe discrepancy of 0.3–2.2 log. The discrepancy was related to gender (6 [26%] of 23 women vs. none of 25 men) (odds ratio 10.9 [95% CI 1.3–90.9], $P=0.027$). Fig. 2B shows a correlation between normalized negative-strand HCV loads in the right lobe (median, 4.9; range, negative to 7.2 log copies/100 ng liver RNA) and the left lobe (median, 5.0; range, negative to 6.3 log copies/100 ng liver RNA) ($r=0.920$; $P<0.001$). A discrepancy of 2.0 log was seen in one (2%) patient (Table 2).

In 38 patients with detectable levels of positive and negative strands in each liver lobe, the ratio of negative- to positive-strand HCV (median, 0.14; range, 0.01–0.81 for R and median, 0.13; range, 0.03–0.45 for L) increased according to negative-strand liver HCV ($r=0.282$; $P=0.086$ for R and $r=0.441$; $P=0.006$ for L) (Fig. 3). The ratio showed no correlation with positive-strand liver HCV ($r=-0.192$; $P=0.248$ for R and $r=-0.097$; $P=0.564$ for L) and circulating HCV ($r=0.154$; $P=0.355$ for R and $r=0.106$; $P=0.527$ for L). Serum HCV RNA loads ranged between 3.1 and 7.6 log copies/mL (median, 6.1), and displayed a relation to the positive-strand liver HCV quantitation ($r=0.604$; $P<0.001$ for R and $r=0.634$; $P<0.001$ for L) and the negative-strand liver HCV quantitation ($r=0.632$; $P<0.001$ for R and $r=0.609$, $P<0.001$ for L).

3.2. Determinants of positive- and negative-strand HCV RNA amounts in the liver

The amounts of positive- and negative-strand HCV in each liver lobe were correlated with patient characteristics including age, gender, mode of infection, duration of infection estimated from years after blood transfusion, serum alanine aminotransferase (ALT) levels, histologic grade and stage, HCV genotypes and detection of HBV DNA in the corresponding liver lobe. An inverse correlation was found between the negative-strand liver HCV

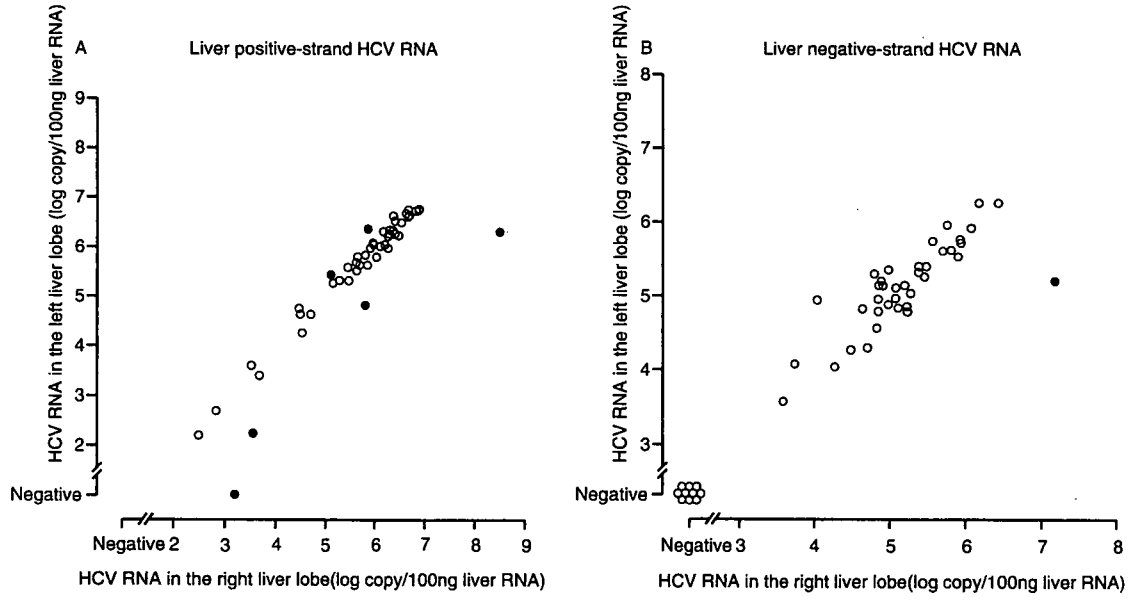

Fig. 2. Correlations between positive-strand HCV RNA levels of the right and left liver lobes ($r=0.950$; $P<0.001$) (A) and between negative-strand HCV RNA levels of each liver lobe ($r=0.920$; $P<0.001$) (B) in the 48 chronic hepatitis C patients. Between-lobe discrepancy of the viral loads was found in six (13%) patients for positive-strand HCV RNA and one (2%) patient for negative-strand HCV RNA (closed circles).

Table 2
Normalized positive- and negative-strand HCV RNA amounts in the right and left liver lobes in 48 chronic hepatitis C patients

Patient	Age (years)	Positive-strand HCV RNA[a]		Negative-strand HCV RNA[a]		HBV DNA[b]	
		Right	Left	Right	Left	Right	Left
1	62	5.96	6.08	5.23	4.80	–	–
2	47	6.88	6.73	6.18	6.26	–	–
3	66	4.48	4.74	3.59	3.58	–	–
4	52	6.68	6.60	5.90	5.53	–	–
5	68	6.38	6.62	5.28	5.04	–	–
6	49	6.04	5.79	4.98	4.89	–	–
7	64	6.11	6.00	4.99	5.36	–	–
8	49	6.54	6.48	5.94	5.72	–	–
9	54	8.51	6.30[c]	7.18	5.20[c]	–	–
10	46	6.30	6.26	5.23	4.86	–	–
11	67	4.49	4.63	4.28	4.04	–	–
12	57	5.80	5.83	5.08	4.97	–	–
13	53	4.53	4.25	–	–	–	–
14	48	5.69	5.62	4.83	4.57	–	–
15	48	5.97	6.04	4.91	5.15	–	–
16	57	5.48	5.30	4.71	4.30	–	–
17	33	6.89	6.76	6.08	5.92	–	+
18	57	5.65	5.79	4.64	4.83	–	–
19	69	3.68	3.38	–	–	–	–
20	64	6.41	6.26	5.11	4.84	–	–
21	59	6.20	6.04	5.38	5.40	–	–
22	56	5.86	6.36[c]	5.76	5.97	–	–
23	67	5.45	5.57	4.86	5.15	–	–
24	38	6.28	6.20	5.81	5.63	–	–
25	48	2.49	2.18	–	–	–	–
26	60	2.83	2.68	–	–	–	–
27	48	5.28	5.30	4.04	4.95	–	–
28	68	5.80	4.80[c]	–	–	–	–
29	44	5.62	5.68	4.85	4.96	–	–
30	43	3.20	–[c]	–	–	–	–
31	58	6.70	6.64	5.46	5.26	–	–
32	56	6.63	6.67	6.43	6.26	–	–
33	45	6.28	5.97	5.20	5.15	–	–
34	50	6.36	6.32	5.93	5.77	+	+
35	63	5.11	5.43[c]	4.49	4.28	–	–
36	70	4.71	4.62	–	–	–	–
37	52	6.83	6.72	4.80	5.30	–	–
38	41	3.56	2.23[c]	–	–	–	–
39	51	6.41	6.52	5.70	5.62	+	+
40	67	5.64	5.51	–	–	–	–
41	61	3.52	3.57	–	–	–	–
42	52	5.91	5.97	5.08	5.11	–	–
43	59	6.18	6.30	5.38	5.32	–	–
44	57	6.48	6.23	5.48	5.40	–	–
45	67	5.15	5.26	3.75	4.08	–	–
46	58	6.68	6.73	5.56	5.75	+	+
47	62	6.30	6.34	4.89	5.20	–	–
48	66	5.85	5.63	4.85	4.79	–	–

[a] Hepatic HCV RNA amounts were normalized to GAPDH mRNA amounts and expressed as log copy/100 ng liver RNA.
[b] Liver HBV DNA was detected by nested PCR using two sets of primers in the surface and X regions, respectively.
[c] Between-lobe differences in the normalized HCV RNA amounts were considered significant according to the intra-assay variance-based criteria.

quantitation and age ($r=-0.322$; $P=0.026$ for R and $r=-0.340$; $P=0.018$ for L). The positive-strand liver HCV quantitation, however, had no relation to age ($r=-0.237$; $P=0.104$ for R and $r=-0.216$; $P=0.140$ for L) (Fig. 4). The amounts of positive- and negative-strand liver HCV did not differ between 38 patients with HCV genotype 1b and 10 patients with genotype 2 (seven with genotype 2a and three with genotype 2b), but were affected by concomitant liver HBV. By using X primers, HBV DNA was detected in both liver lobes in three patients and only in the left lobe in another patient. None of the patients tested positive for liver HBV DNA using surface primers. The four