差があった因子は HBV-DNA 量だけであった。6 Log copy/mL 未満、6 Log copy/mL 以上の 2 群に分けると、HBsAg 陰性化群では 72% vs 29%、HBsAg 陽性群では 12% vs 89%であり、NA 投与開始時の HBV-DNA 量が少ない症例で有意に HBsAg 陰性化する症例が多いことが示された (P = 0.001)。このことは kaplan-Meier plot による累積 HBsAg 陰性化率でも確認された (P = 0.015)。

6．発癌症例の解析

全 94 例中 5 例 (5.3%；年率 0.6%) に発癌を認めた。内訳は HBeAg 陰性 39 例中 2 例 (5.1%；年率 0.6%)、HBeAg 陽性例 55 例中 3 例 (5.5%；年率 0.6%) であった。発癌例の背景因子を解析すると年齢のみが有意な因子であり、発癌例 5 例中 4 例 (80%) が 60 才以上であり、非発癌例 79 例中 16 例 (18%) に比し、有意に高齢者が多かった(P = 0.01)。Kaplan-Meier plot でも同様の結果であった (P = 0.01)。

7．死亡症例の解析

94 例中 4 例 (4.3%) で死亡を認め、4 例全例が HBeAg 陽性患者であった。肝関連死は HCC の 1 例(1%)のみであり、他 3 例の死因は胃癌、膵癌、原因不明であった。

D. 考察

NA 投与後約 9 年間の経過で drug-free となった症例の割合は、HBeAg 陰性例で 39 例中 17 例 (44%)、陽性例で 55 例中 8 例(15%)であった。したがって、HBeAg 陰性例の半数以上、HBeAg 陽性例の多くは NA の服用を続けざるを得ない状況にあるといえる。HBeAg 陰性者においては半数弱で drug-free にすることが可能であることが判明した。

HBeAg 陰性症例ではほとんどの症例 (95%) で中止可能基準を満たしたが、HBeAg 陽性症例で中止可能基準を満たしたのはわずか 27%であった。Ono らは 4 年間の ETV 投与により、HBeAg 陽性患者の 93%で HBV-DNA が陰性化したが、seroconversion したのは 38%であったことを報告しており、HBeAg 陽性症例では NA 投与により、seroconvsersion を達成するのは一部の症例にとどまるようである。HBeAg 陽性症例のうち、中止可能基準を満たすことに関与する因子は NA 投与前の HBV-DNA が少ない症例であった。すなわち、ウイルス量の多い HBeAg 陽性症例に対して NA を投与開始した場合には中止することが困難であると考えられる。

NA 治療開始前の因子では血清 ALT 値が高い症例で再燃が有意に少ないことが判明した。これは NA 開始時に HBV に対する強い免疫応答が惹起されていることを示唆し、免疫応答期 immune clearance phase から低増殖期 low replicative phase に向かっている過程であったのかもしれない。

再燃症例 15 例のうち、27%は ALT 500 IU/L 以上の shub を起こしたが、肝不全とはならなかった。NA 製剤の治験、臨床研究ではある一定の期間 NA 製剤を投与した後、中止しているが、肝不全となった症例はほとんど報告されていない。一方、HBV 再活性化時には致死的な肝不全の発症が多く報告されている。この違いは、リツキサンなどの化学療法による免疫系の変動が大きく関与していると考えられる。NA 製剤中止後の再燃においては免疫が正常に機能しているため、肝不全に陥ることが少ないのかもしれない。ただ、ガイドラインでも示されているように、F3、肝硬変など肝予備能が低下している場合には注意が必要である。

HBsAg が陰性化した割合は HBeAg 陰性で年率 1.4%、HBeAg 陽性で年率 0.4%、全体で年率 0.8%であった。HBsAg 陰性化に関与する因子は NA 投与前の HBV-DNA が少ない症例であった。NA 投与前の HBsAg 量の少ない症例で HBsAg 陰性化

が高率におこることが予想されるが、本研究では有意差は認められなかった。

発癌はHBeAg陰性で年率0.6%、HBeAg陽性で年率0.6%、全体で年率0.6%に認めた。発癌例の背景因子の解析では年齢のみが有意な因子であり、60才以上で有意に発癌を多かった。これまで報告されているHBV-DNA量や肝線維化は有意差がなかった。これは発癌症例数が少ないことに起因しているかもしれない。

E. 結論

NAを投与されたB型慢性肝炎患者94例について、その長期経過とNAが中止可能となる因子について検討し、以下の成績を得た。

1) 約9年間の経過観察で、drug-freeになる症例の割合はHBeAg陰性例で44%、陽性例で15%であった。

2) NA投与開始時HBV-DNA量低値例でNA中止可能となる症例が多く認められた。

3) NA投与開始時ALT値高値例でNA中止後の再燃が少なかった。

4) HBsAg陰性化は年率0.8%、発癌は年率0.6%で認められた。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

1) Kagawa T, Oka A, Kobayashi Y, Hiasa Y, Kitamura T, Sakugawa H, Adachi Y, Anzai K, Tsuruya K, Arase Y, Hirose S, Shiraishi K, Shiina T, Sato T, Ting W, Tanaka M, Hayashi H, Kawabe N, Robinson PN, Zemojtel T, Mine T. Recessive inheritance of population-specific intronic LINE-1 insertion causes a Rotor syndrome phenotype. Hum Mutat. 2015; 36(3): 327-32.

2) Shomura M, Kagawa T, Shiraishi K, Hirose S, Arase Y, Koizumi J, Mine T. Skin toxicity predicts efficacy to sorafenib in patients with advanced hepatocellular carcinoma. World J Hepatol. 2014; 6(9): 670-6.

3) Kagawa T, Orii R, Hirose S, Arase Y, Shiraishi K, Mizutani A, Tsukamoto H, Mine T. Ursodeoxycholic acid stabilizes the bile salt export pump in the apical membrane in MDCK II cells. J Gastroenterol. 2014; 49(5): 890-9.

2. 学会発表

1) Kagawa T, Anzai K, Tsuruya K, Arase Y, Hirose S, Shiraishi K, Mine T. Contribution of polymorphic LINE-1 retrotransposon insertion in SLCO1B3 gene to susceptibility to drug-induced cholestasis. 65th Annual Meeting of the American Association for the Study of Liver Diseases. Boston, 2014.

2) Tsuruya K, Kamiya A, Chikada H, Anzai K, Arase Y, Hirose S, Kagawa T, Mine T. Charachterization and expansion mechanisms of human iPS cell-derived hepatic progenitor-like cells. 65th Annual Meeting of the American Association for the Study of Liver Diseases. Boston, 2014.

3) 長田成彦、加川建弘、峯 徹哉. B型肝炎の治療-B型慢性肝炎における核酸アナログ中止症例の検討. 第100回日本消化器病学会総会. 東京 2014年

H. 知的財産権の出願・登録状況

(※予定を含む)

1. 特許取得：なし
2. 実用新案登録：なし
3. その他：なし

Ⅲ．研究成果の刊行に関する一覧表

平成26年度　研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻・号	ページ	出版年
Matsumoto A, Yatsuhashi H, Nagaoka S, Suzuki Y, Hosaka T, Tsuge M, Chayama K, Kanda T, Yokosuka O, Nishiguchi S, Saito M, Miyase S, Kang JH, Shinkai N, Tanaka Y, Umemura T, Tanaka E	Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleos(t)ide analogue treatment in patients with chronic hepatitis B	Hepatol Res			in press
Kamijo N, Matsumoto A, Umemura T, Shibata S, Ichikawa Y, Kimura T, Komatsu M, Tanaka E	Mutations of Pre-core and BCP Before and After HBeAg Seroconversion	World J Gastroenterol	21(2)	541-548	2015
Tanaka E, Matsumoto A	Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B	Hepatol Res	44 (1)	1-8	2014
Nishida N, Sawai H, Kashiwase K, Minami M, Sugiyama M, Seto WK, Yuen MF, Posuwan N, Poovorawan Y, Ahn SH, Han KH, Matsuura K, Tanaka Y, Kurosaki M, Asahina Y, Izumi N, Kang JH, Hige S, Ide T, Yamamoto K, Sakaida I, Murawaki Y, Itoh Y, Tamori A, Orito E, Hiasa Y, Honda M, Kaneko S, Mita E, Suzuki K, Hino K, Tanaka E, Mochida S, Watanabe M, Eguchi Y, Masaki N, Murata K, Korenaga M, Mawatari Y, Ohashi J, Kawashima M, Tokunaga K, Mizokami M	New susceptibility and resistance HLA-DP alleles to HBV-related diseases identified by a trans-ethnic association study in Asia	PLoS One	9 (2)	e86449	2014
Okuhara S, Umemura T, Joshita S, Shibata S, Kimura T, Morita S, Komatsu M, Matsumoto A, Yoshizawa K, Katsuyama Y, Ota M, Tanaka E	Serum levels of interleukin-22 and hepatitis B core-related antigen are associated with treatment response to entecavir therapy in chronic hepatitis B.	Hepatol Res	44 (10)	E172-180	2014

Morita S, Matsumoto A, Umemura T, Shibata S, Kamijo N, Ichikawa Y, Kimura T, Joshita S, Komatsu M, Yoshizawa K, Tanaka E	Characteristics and prediction of hepatitis B e-antigen negative hepatitis following seroconversion in patients with chronic hepatitis B.	Hepatol Res	44 (10)	E45-53	2014
Posuwan N, Payungporn S, Tangkijvanich P, Ogawa S, Murakami S, Iijima S, Matsuura K, Shinkai N, Watanabe T, Poovorawan Y, Tanaka Y.	Genetic association of human leukocyte antigens with chronicity or resolution of hepatitis B infection in thai population.	PLoS One	9(1)	e86007	2014
Yamada R, Hiramatsu N, Oze T, Morishita N, Harada N, Yakushijin T, Iio S, Doi Y, Yamada A, Kaneko A, Hagiwara H, Mita E, Oshita M, Itoh T, Fukui H, Hijioka T, Katayama K, Tamura S, Yoshihara H, Imai Y, Kato M, Miyagi T, Yoshida Y, Tatsumi T, Kasahara A, Hamasaki T, Hayashi N, Takehara T; the Osaka Liver Forum.	Impact of alpha-fetoprotein on hepatocellular carcinoma development during entecavir treatment of chronic hepatitis B virus infection.	J Gastroenterol			in press
Suzuki F, Hosaka T, Suzuki Y, Akuta N, Sezaki H, Hara T, Kawamura Y, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Watahiki S, Mineta R, Kumada H	Long-term efficacy and emergence of multidrug resistance in patients with lamivudine-refractory chronic hepatitis B treated by combination therapy with adefovir plus lamivudine.	J Gastroenterol	49	1094-1104	2014
Hara T, Suzuki F, Kawamura Y, Sezaki H, Hosaka T, Akuta N, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Watahiki S, Kumada H	Long-term entecavir therapy results in falls in serum hepatitis B surface antigen levels and seroclearance in nucleos(t)ide-naive chronic hepatitis B patients.	J Viral Hepat	21	802-808	2014

Suzuki F, Akuta N, Suzuki Y, Kawamura Y, Hosaka T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Watahiki S, Mineta R, Suzuki Y, Kumada H	Virologic breakthrough in a patient with chronic hepatitis B by combination treatment with tenofovir disoproxil fumarate and entecavir.	Drug Des Devel	8	869-873	2014
Akuta N, Suzuki F, Kobayashi M, Hara T, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Saitoh S, Ikeda K, Kumada H	Correlation Between Hepatitis B Virus Surface Antigen Level and Alpha-Fetoprotein in Patients Free of Hepatocellular Carcinoma or Severe Hepatitis.	J Med Virol	86	131-138	2014
Tanaka M, Suzuki F, Seko Y, Hara T, Kawamura Y, Sezaki H, Hosaka T, Akuta N, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H	Renal dysfunction and hypophosphatemia during long-term lamivudine plus adefovir dipivoxil therapy in patients with chonic hepatitis B.	J Gastroenterol	49	470-480	2014
Kobayashi M, Hosaka T, Suzuki F, Akuta N, Sezaki H, Suzuki Y, Kawamura Y, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Miyakawa Y, Kumada H	Seroclearance rate of hepatitis B surface antigen in 2,112 patients with chronic hepatitis in Japan during long-term follow-up.	J Gastroenterol	49	538-546	2014
Hosaka T, Suzuki F, Kobayashi M, Fukushima T, Kawamura Y, Sezaki H, Akuta N, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Kobayashi M, Kumada H	HLA-DP genes polymorphisms associate with hepatitis B surface antigen kinetics and seroclearance during nucleo(s)ide analogue therapy.	Liver Int		Epub ahead of print	2014
Bae SK, Yatsuhashi H, Takahara I, Tamada Y, Hashimoto S, Motoyoshi Y, Ozawa E, Nagaoka S, Yanagi K, Abiru S, Komori A, Ishibashi H.	Sequential occurrence of acute hepatitis B among members of a high school Sumo wrestling club.	Hepatol Res	44(10)	E267-272	2014
Tsutsui H, Nishiguchi S.	Importance of Kupffer cells in the development of acute liver injuries in mice.	Int J Mol Sci	15(5)	7711-30	2014

Enomoto H, Aizawa N, Nakamura H, Sakai Y, Iwata Y, Tanaka H, Ikeda N, Aoki T, Yuri Y, Yoh K, Hashimoto K, Ishii A, Takashima T, Iwata K, Saito M, Imanishi H, Iijima H, Nishiguchi S.	An Increased Ratio of Glycated Albumin to HbAlc Is Associated with the Degree of Liver Fibrosis in Hepatitis B Virus-Positive Patients.	Gastroenterol Res Pract			2014
Akamatsu S, Hayes CN, Tsuge M, Miki D, Akiyama R, Ochi H, Hiraga N, Imamura M, Aikata H, Kawaoka T, Kawakami Y, Chayama K.	Differences in serum microRNA profiles in hepatitis B and C virus infection.	J Infect			in press
Kohno T, Tsuge M, Murakami E, Hiraga N, Abe H, Miki D, Imamura M, Ochi H, Hayes CN and Chayama K.	Human microRNA hsa-miR-1231 suppresses hepatitis B virus replication by targeting core mRNA.	J Viral Hepat			in press
Huang YW, Takahashi S, Tsuge M, Chen CL, Wang TC, Abe H, Hu JT, Chen DS, Yang SS, Chayama K and Kao JH	On-treatment low serum HBV RNA level predicts initial virological response in chronic hepatitis B patients receiving nucleoside analogue therapy.	Antivir Ther			in press
Nakamura M, Kanda T, Nakamoto S, Haga Y, Sasaki R, Jiang X, Yasui S, Arai M, Yokosuka O.	Reappearance of serum HBV DNA in patients with hepatitis B surface antigen seroclearance.	Hepatology		Epub ahead of print	2015
Yasui S, Fujiwara K, Nakamura M, Miyamura T, Yonemitsu Y, Mikata R, Arai M, Kanda T, Imazeki F, Oda S, Yokosuka O.	Virological efficacy of combination therapy with corticosteroid and nucleoside analogue for severe acute exacerbation of chronic hepatitis B.	J Viral Hepat	22(2)	94-102	2015
Sarkar N, Panigrahi R, Pal A, Biswas A, Singh SP, Kar SK, Bandopadhyay M, Das D, Saha D, Kanda T, Sugiyama M, Chakrabarti S, Banerjee A, Chakravarty R.	Expression of microRNA-155 correlates positively with the expression of Toll-like receptor 7 and modulates hepatitis B virus via C/EBP-β in hepatocytes.	J Viral Hepat		Epub ahead of print	2015

Nakamoto S, Kanda T, Nakaseko C, Sakaida E, Ohwada C, Takeuchi M, Takeda Y, Mimura N, Iseki T, Wu S, Arai M, Imazeki F, Saito K, Shirasawa H, Yokosuka O.	Reactivation of hepatitis B virus in hematopoietic stem cell transplant recipients in Japan: efficacy of nucleos(t)ide analogues for prevention and treatment.	Int J Mol Sci	15(11)	21455-67	2014
Jiang X, Kanda T, Wu S, Nakamura M, Miyamura T, Nakamoto S, Banerjee A, Yokosuka O.	Regulation of microRNA by hepatitis B virus infection and their possible association with control of innate immunity.	World J Gastroenterol	20(23)	7197-206	2014
Orito E, Hasebe C, Kurosaki M, Osaki Y, Jyoko K, Watanabe H, Kimura H, Nishijima N, Kusakabe A, Izumi N.	Risk of hepatocellular carcinoma in cirrhotic HBV patients during nucleot(s)ide analogues therapy.	Hepatol Res			in press
Ito K, Yotsuyanagi H, Yatsuhashi H, Karino Y, Takikawa Y, Saito T, Arase Y, Imazeki F, Kurosaki M, Umemura T, Ichida T, Toyoda H, Yoneda M, Mita E, Yamamoto K, Michitaka K, Maeshiro T, Tanuma J, Tanaka Y, Sugiyama M, Murata K, Masaki N, Mizokami M.	Risk factors for long-term persistence of serum hepatitis B surface antigen following acute hepatitis B virus infection in Japanese adults.	Hepatology	59	89-97	2014

Ⅳ．研究成果の刊行物・別刷

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Original Article

Factors associated with the effect of interferon-α sequential therapy in order to discontinue nucleoside/nucleotide analog treatment in patients with chronic hepatitis B

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Aim: The factors associated with the outcome of sequential therapy with interferon-α (IFN-α) in order to halt nucleoside/nucleotide analog (NUC) maintenance treatment for chronic hepatitis B were analyzed.

Methods: A total of 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy for cessation of NUC were enrolled retrospectively. The subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 24 weeks. Natural IFN-α of 6-MU doses was administrated three times a week. A successful response to NUC/IFN-α sequential therapy was defined as serum hepatitis B virus (HBV) DNA below 4.0 log copies/mL, serum alanine aminotransferase (ALT) below 30 IU/L, and hepatitis B e-antigen negativity at 24 months after completing the treatment.

Results: Multivariate analysis revealed that hepatitis B surface antigen (HBsAg) of 3.0 log U/mL or more ($P < 0.002$) and hepatitis B core-related antigen (hepatitis B core-related antigen [HBcrAg]) of 4.5 log U/mL or more ($P < 0.003$) at the start of IFN-α administration were significant factors associated with a 24-month non-response. Maximal levels of ALT and HBV DNA during the follow-up period after completing IFN-α therapy were significantly related ($P < 0.001$), and receiver-operator curve analysis showed that both maximal ALT ($P < 0.001$) and HBV DNA ($P < 0.001$) were significantly related to the final 24-month response.

Conclusion: The combinational use of HBsAg and HBcrAg levels may be useful to predict the 24-month outcome of NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during post-treatment follow-up may also help monitor responses to IFN-α sequential therapy.

Key words: hepatitis B core-related antigen, hepatitis B surface antigen, interferon-α, nucleoside/nucleotide analogs, sequential therapy

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a widespread health problem with an estimated 350–400 million carriers worldwide. Prolonged infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma (HCC).[1-3] Currently available antiviral treatments for hepatitis B include nucleoside/nucleotide analogs (NUC) and interferon-α (IFN-α).[4] NUC are p.o. administrated and are associated with low rates of adverse effects. Although treatment with NUC, such as lamivudine (LVD), adefovir dipivoxil and entecavir (ETV), induces virological and biochemical responses in most patients, NUC therapy also carries the risk of drug resistance. Furthermore, patients with hepatitis B are required to undergo extended

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treatment with NUC because early discontinuance often leads to relapse.[5,6] In contrast, the remission of chronic hepatitis B by IFN-α is prolonged, but is achieved only in a small percentage of patients.

Serfaty *et al.*[7] conducted a pilot study on sequential therapy using LVD and IFN-α and concluded that this treatment could induce a sustained virological response in patients with chronic hepatitis B who did not respond to IFN-α alone. However, ensuing reports[8-12] were unable to confirm such a cooperative effect. Because the clinical backgrounds of the enrolled patients also differed among the above reports, it has become necessary to clarify the factors associated with the outcome of IFN-α sequential therapy in order to estimate its clinical significance.

We previously analyzed patients with chronic hepatitis B who ceased NUC therapy and showed that lower hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) levels were associated with a favorable clinical outcome in subjects negative for hepatitis B e-antigen (HBeAg) and HBV DNA at NUC discontinuation.[13,14] Although we identified patients in whom NUC could be safely halted with high reliance, such patients accounted for a relatively minor percentage. Therefore, we conducted the present study to analyze the effect of IFN-α sequential therapy on successfully stopping NUC.

This report retrospectively analyzes the factors associated with outcome of IFN-α sequential therapy following NUC treatment. As the subjects were followed long term, treatment responses at 24 months after stopping IFN-α were evaluated and compared with those at 6 and 12 months.

METHODS

Patients

A TOTAL OF 50 patients with chronic hepatitis B who underwent IFN-α sequential therapy in order to halt NUC therapy between May 2002 and September 2010 were enrolled. Subjects received NUC plus IFN-α for 4 weeks followed by IFN-α alone for 20 weeks (Fig. 1). Natural IFN-α (Sumiferon; Sumitomo Dainippon Pharma, Tokyo, USA) at a dose of 6 MU was administrated three times a week. Doses were reduced to 3 MU during exceptional circumstances, such as side-effects. All patients completed 24 weeks of IFN-α administration and received over 80% of the scheduled dose. Patients were recruited retrospectively from eight hospitals across Japan (Shinshu University Hospital, National Hospital Organization Nagasaki Medical Center, Toranomon Hospital, Hiroshima University Hospital, Chiba University Hospital, The Hospital of Hyogo College of Medicine, Kumamoto Shinto General Hospital,and Teine Keijinkai Hospital). The demographic data of the subjects are presented in Table 1. The median age at NUC cessation was 35 years. Approximately three-fourths of the patients were men. Genotype C HBV was predominant as has earlier been reported for Japan.[15] Eighty-six percent of patients began NUC therapy with LVD and 14% did so with ETV. The duration of NUC administration ranged from 4 to 121 months. The follow-up period was defined as the point of stopping IFN-α administration up until the last visit or to when NUC were re-administrated due to reactivation of hepatitis B. NUC were recommenced in 25 (50%) of the 50 patients enrolled. Among them, 17 were treated before judgment of the 24-month response to sequential therapy. All patients requiring re-administration

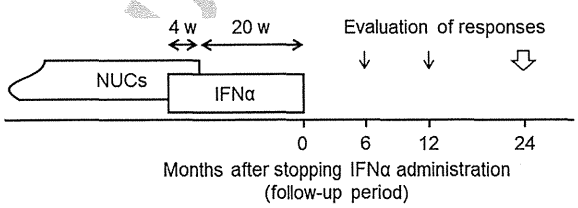

Figure 1 Experimental design of the present study. IFN, interferon; NUC, nucleoside/nucleotide analog; w, weeks.

Table 1 Demographic data of 50 enrolled patients

Characteristic	Value
Age at start of NUC administration (years)†	34 (21–57)
Age at end of NUC administration (years)†	35 (22–62)
Sex (male : female)	38:12
Genotype (B : C : undetermined)	3:36:11
NUC at start (LVD : ETV)	43:7
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	40:8:1:1
Duration of NUC administration (months)†	6 (4–121)
HBeAg positivity at start of NUC‡	70% (35/50)
HBeAg positivity at end of NUC‡	42% (21/50)
Follow-up period after stopping IFN-α administration (months)†	28 (2–102)
Patients requiring re-administration of NUC‡	50% (25/50)
Patients developing HCC‡	0% (0/50)

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

of NUC possessed alanine aminotransferase (ALT) levels of over 80 IU/L and HBV DNA levels of over 5.8 log copies/mL at or just before the point of NUC re-continuation, which fulfilled the established requirements for restarting NUC.[13,14,16]

Hepatitis B surface antigen was confirmed to be positive on at least two occasions at least 6 months apart in all patients before NUC treatment. Tests for hepatitis C virus and HIV antibodies were all negative. Patients complicated with HCC or signs of hepatic failure at the cessation of NUC administration were excluded from the study. No such complications were observed during follow up.

With few exceptions, patients were seen at least once a month during the first year of follow up, at least once every 3 months during the second year and at least once every 6 months afterwards. No patient developed HCC or hepatic failure during the follow-up period. Stored serum samples were kept frozen at −20°C or below until assayed. This study was approved by the ethics committees of all participating institutions (approval reference 1117 for Shinshu University Hospital, 24085 for National Hospital Organization Nagasaki Medical Center, 758 for Toranomon Hospital, 321 for Hiroshima University Hospital, 934 and 977 for Chiba University Hospital, 779 for The Hospital of Hyogo College of Medicine, 411 for Kumamoto Shinto General Hospital, and "Analysis of efficacy of IFN- to stop NUC in patients with chronic hepatitis B" for Teine Keijinkai Hospital).

Hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg and antibody to HBeAg, were tested using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan; Fujirebio, Tokyo, Japan; and/or Sysmex, Kobe, Japan) at each hospital. Quantitative measurement of HBsAg[17] was performed using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex (Kobe, Japan). The assay had a quantitative range of ~~=1.5~~ to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[18] with a quantitative range of 2.1–9.0 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal, and no signal detection was regarded as a negative signal. Six HBV genotypes (A–F) were evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al.*[19]

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio) as described previously.[20,21] The HBcrAg assay measures all antigens transcribed and translated from the precore and core genes of the HBV genome, which include hepatitis B e, core and p22cr antigens.[14,20] HBcrAg concentration was calculated based on a standard curve generated using recombinant pro-HBeAg. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL, with a quantitative range set at 3.0–6.8 log U/mL.

Evaluation of response to NUC/IFN-α sequential therapy

The clinical conditions of a successful response to NUC/IFN-α sequential therapy were set at serum HBV DNA below 4.0 log copies/mL, serum ALT below 30 IU/L and negative HBeAg, according to established Japanese guidelines in which patients who meet these conditions are not recommended to start antiviral therapy.[22] We assessed the final response at approximately 24 months after completing IFN-α sequential therapy and compared results to those at 6 and 12 months after the treatment.

Statistical analyses

Fisher's exact and Pearson's χ^2-tests were adopted to test for differences between subgroups of patients. The Mann–Whitney *U*-test was employed to compare continuous data. Each cut-off value was decided using ~~receiver-operator curve~~ (ROC) analysis, and results were evaluated by measuring the area under the ROC (AUC). Multivariate analysis was performed using a logistic model for the 24-month response to NUC/IFN-α sequential therapy. Correlations between maximal values of ALT and HBV DNA were calculated using Spearman's rank correlation coefficient test. The non-relapse rate was analyzed by the Kaplan–Meier method.

All tests were performed using the IBM SPSS Statistics Desktop for Japan version 19.0 (IBM Japan, Tokyo, Japan). $P < 0.05$ was considered to be statistically significant.

RESULTS

Factors associated with the 24-month response to NUC/IFN-α sequential therapy

OF THE 50 patients enrolled, 18 were judged as responders at 24 months after completing IFN-α sequential therapy (i.e. 24-month responders), while the

remaining 32 were classified as 24-month non-responders. The clinical backgrounds of both groups are compared in T2 Table 2. The median age at NUC commencement and sex distribution did not differ remarkably between the groups. Genotype C was similarly predominant. The types of NUC administrated at the start and end of treatment were comparable between the groups, but the duration of NUC administration was significantly longer in responders. Re-administration of NUC due to aggravation of hepatitis B before judgment of the 24-month response was observed in approximately half of the 32 non-responders. After the final evaluation at 24 months, re-continuation of NUC was seen in only one of the 18 responders versus roughly half of the 15 non-responders who had previously not required it. The follow-up period was significantly longer in responders because observation was discontinued when NUC were re-administrated.

Biochemical and virological markers were compared between 24-month responders and non-responders at the start of NUC, at the start of IFN-α and at the end of IFN-α T3 (Table 3). Positivity for the HBeAg was significantly lower in responders at all time points. HBsAg and HBcrAg levels did not differ between the groups at the start of NUC, but became significantly lower in responders at the start and end-points of IFN-α administration. A significant difference in HBV DNA level was seen between the groups at the end of IFN-α administration only. ALT levels did not differ between the groups at any point.

Multivariate analysis revealed that HBsAg and HBcrAg levels of 3.0 or more and 4.5 log U/mL or more, respectively, at the start of IFN-α administration were significant factors associated with a 24-month non-response to T4 NUC/IFN-α sequential therapy (Table 4). The factors adopted for this logistic model were as follows: age at end of NUC of 37 years or more, duration of NUC administration of 18 months or more, sex, type of NUC at start, HBV genotype, HBeAg positivity at the start of IFN-α, HBsAg level at the start of IFN-α of 3.0 log IU/mL or more, and HBcrAg level at the start of IFN-α of 4.5 log U/mL or more. The corresponding cut-off values for each factor were determined by ROC analysis.

Of the 50 patients enrolled, 23 (46%) had HBsAg of 3.0 log IU/mL or more and HBcrAg of 4.5 log U/mL or more, 27 (54%) had HBsAg of less than 3.0 log IU/mL or HBcrAg of less than 4.5 log U/mL, and none had HBsAg of less than 3.0 log IU/mL and HBcrAg of less than 4.5 log U/mL at the start of IFN-α administration. Whereas none of the 23 patients with the highest HBsAg and HBcrAg levels were responders, 18 (67%) of the remaining 27 patients responded to NUC/IFN-α sequential therapy ($P = 0.005$).

Comparison of responses to NUC/IFN-α sequential therapy at different time points

We assessed the responses to NUC/IFN-α sequential therapy at 6 and 12 months after completing IFN-α administration using the same criteria as those for determining the 24-month outcome. Responses were in 78% agreement ($P < 0.001$) between 6 and 24 months and 80% agreement ($P < 0.001$) between 12 and 24 months.

Prediction of response to NUC/IFN-α sequential therapy using maximal levels of ALT and HBV DNA

The maximal levels of ALT and HBV DNA during follow up were found to be significantly related ($r = 0.777$, $P < 0.001$). ROC analysis showed that both maximal ALT

Table 2 Comparison of clinical backgrounds between 24-month responders and non-responders

Clinical background	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	P
Age at start of NUC (years)†	36 (21–56)	34 (21–57)	0.486
Sex (male : female)	15:3	23:9	0.497
Genotype (B:C:undetermined)	1:16:1	2:20:10	0.101
NUC at start (LVD : ETV)	16:2	27:5	1.000
NUC at end (LVD : ETV : LAM + ADV : ETV + ADV)	16:2:0:0	24:6:1:1	0.610
Duration of NUC administration (months)†	51 (5–121)	5 (4–72)	0.001
Follow-up period after stopping IFN-α administration (months)†	30 (23–102)	22 (2–81)	0.014
Re-administration of NUC before judging 24-month response‡	0% (0/18)	53% (17/32)	<0.001
Re-administration of NUC after judging 24-month response‡	6% (1/18)	47% (7/15)	0.012

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ADV, adefovir dipivoxil; ETV, entecavir; HBeAg, hepatitis B e-antigen; HCC, hepatocellular carcinoma; IFN, interferon; LAM, lamivudine; LVD, lamivudine; NUC, nucleoside/nucleotide analog.

Table 3 Comparison of ALT level and viral markers between 24-month responders and non-responders at the time points of starting NUC administration, starting IFN-α administration and stopping IFN-α administration

ALT/viral marker	24-month responders ($n = 18$)	24-month non-responders ($n = 32$)	P
At start of NUC administration			
ALT (IU/L)†	242 (32–2274)	281 (22–1044)	0.872
HBeAg‡	44% (8/18)	84% (27/32)	0.008
HBV DNA (log copies/mL)†	8.0 (<2.1–>9.0)	7.8 (<2.1–>9.0)	0.866
HBsAg (log IU/mL)†	3.5 (1.8–4.9)	3.5 (2.5–4.4)	1.000
HBcrAg (log U/mL)†	>6.8 (3.7–>6.8)	>6.8 (<3.0–>6.8)	0.121
At start of IFN-α administration			
ALT (IU/L)†	29 (12–103)	29 (12–111)	0.779
HBeAg‡	11% (2/18)	59% (19/32)	0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–3.9)	<2.1 (neg.–4.8)	0.142
HBsAg (log IU/mL)†	2.9 (1.5–4.1)	3.7 (2.5–4.3)	0.028
HBcrAg (log U/mL)†	3.6 (<3.0–5.9)	5.6 (<3.0–>6.8)	0.002
At end of IFN-α administration			
ALT (IU/L)†	25 (10–48)	28 (12–134)	0.384
HBeAg‡	6% (1/18)	59% (19/32)	<0.001
HBV DNA (log copies/mL)†	<2.1 (neg.–4.1)	4.6 (<2.1–>9.0)	<0.001
HBsAg (log IU/mL)†	2.8 (1.9–4.0)	3.6 (2.6–4.7)	0.007
HBcrAg (log U/mL)†	3.4 (<3.0–5.5)	5.5 (<3.0–>6.8)	0.017

†Data are expressed as the median (range).
‡Data are expressed as a positive percentage (positive number/total number).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

Table 4 Multivariate analysis of factors associated with 24-month non-responders to NUC/IFN-α sequential therapy

Selected factor	Odds ratio	95% CI	P
HBsAg ≥3.0 log IU/mL at start of IFN-α	17.7	2.9–108.2	0.002
HBcrAg ≥4.5 log U/mL at start of IFN-α	15.0	2.5–88.6	0.003

CI, confidence interval; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; IFN, interferon; neg., negative; NUC, nucleoside/nucleotide analog.

and HBV DNA levels were significantly associated with the treatment response (Fig. 2), with an AUC for each parameter of over 0.8. The cut-off values providing the highest significance in ROC analysis were 128 IU/L for ALT and 4.5 log copies/mL for HBV DNA. The existence of a second cut-off value was also identified for HBV DNA (6.0 log copies/mL) to discriminate between 24-month responders and non-responders. These results indicated that patients reaching a maximal ALT level of over 128 IU/L or maximal HBV DNA level of over 6.0 log copies/mL during post-treatment follow up were likely to be non-responders.

Lastly, we analyzed the changes in cumulative non-relapse rate of hepatitis B during and after IFN-α administration by tentatively defining relapse as ALT level exceeding 128 IU/L during follow up. We selected maximal ALT instead of maximal HBV DNA because: (i) the inflection point to distinguish a response was clear for maximal ALT but ambiguous for maximal HBV DNA; (ii) the value for "sensitivity + specificity − 1" as calculated by ROC analysis was larger for maximal ALT (7.5 vs 6.5); and (iii) the maximal levels of ALT and HBV DNA were closely associated, and thus ALT values were considered to represent those of HBV DNA. The cumulative non-relapse rate decreased rapidly after completely halting NUC until just prior to 6 months after stopping IFN-α and then was seen to plateau until the study end-point (Fig. 3). This suggests that the recurrence of hepatitis associated with a 24-month non-response can be expected to occur primarily during the first 6 months after stopping IFN-α administration.

DISCUSSION

THE COOPERATIVE EFFECT of NUC/IFN-α sequential therapy has been controversial.[7-12] Enomoto *et al.*[10] first analyzed the results of ETV/IFN-α sequential therapy in patients with HBeAg positive chronic hepatitis B and detected several differences. Although their results were negative, they witnessed that patients who had achieved HBeAg

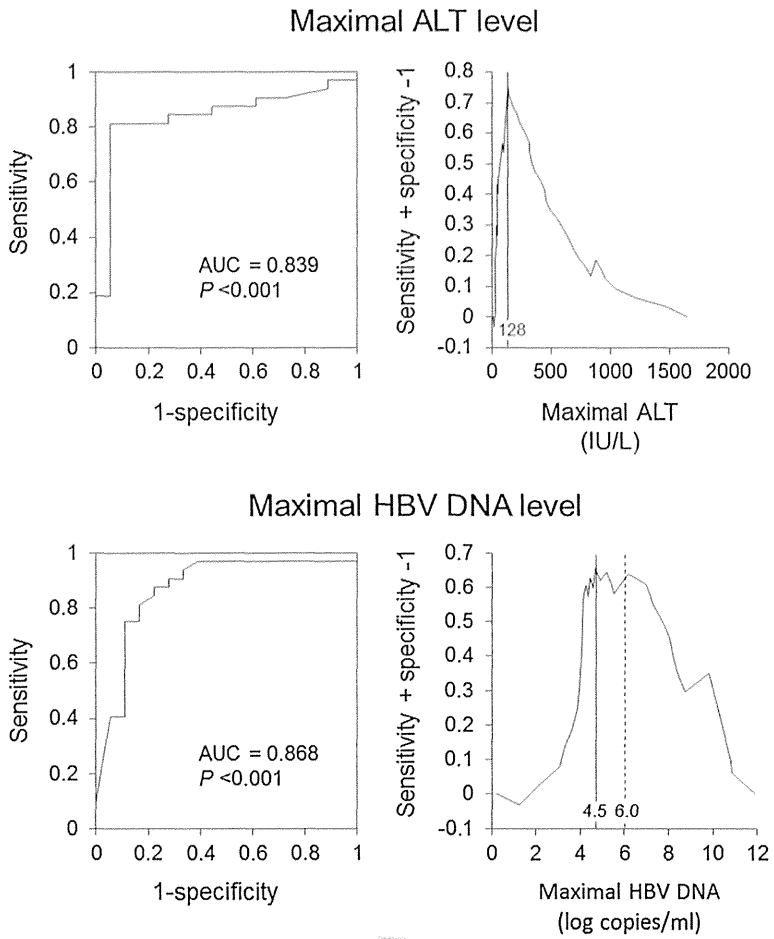

Figure 2 ~~Receiver–operator curve~~ analysis of maximal alanine aminotransferase (ALT) and hepatitis B virus (HBV) DNA levels to discriminate between 24-month responders and non-responders. Vertical solid lines indicate the actual values of markers corresponding to main inflection points and the vertical broken line indicates the actual value of the marker corresponding to a second inflection point; AUC, area under the ~~receiver–operator~~ curve.

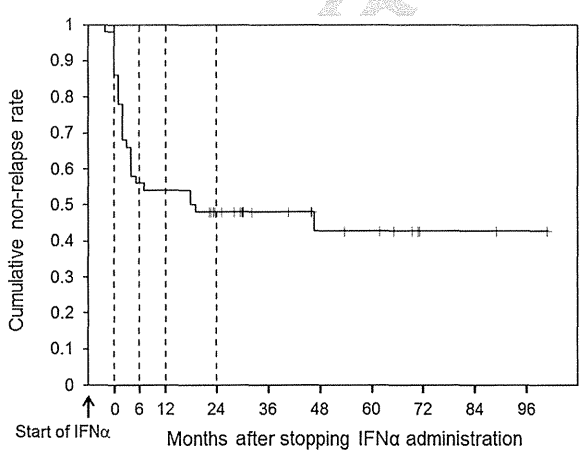

Figure 3 Kaplan–Meier analysis of the non-relapse rate after ~~starting~~ interferon (IFN)-α administration by defining relapse of hepatitis B as alanine aminotransferase (ALT) level exceeding 128 IU/L.

seroconversion by the time of IFN-α commencement experienced a significantly higher sustained virological response rate than those in whom the HBeAg persisted. Thus, it appeared beneficial to further clarify the factors associated with the response to NUC/IFN-α sequential therapy.

The present study analyzed the factors associated with a long-term response to IFN-α sequential therapy in order to safely discontinue NUC therapy. All patients were treated with natural IFN-α for 6 months and followed for at least 24 months after completing the sequential therapy, with the exception of those who required re-administration of NUC due to aggravation of hepatitis B. The type and duration of NUC administration were not fixed in this study because IFN-α sequential therapy was implemented to discontinue NUC in patients who were undergoing maintenance treatment. Although a prospective study would have been ideal to elucidate the factors associated with

IFN-α sequential therapy outcome, we undertook this retrospective trial because no variables have been sufficiently analyzed to date. Furthermore, we were able to address the long-term response to IFN-α sequential therapy in relation to the results of earlier retrospective studies. It has been reported that pegylated IFN-α (PEG IFN-α) provides a higher HBV response rate than does conventional IFN-α.[23] Therefore, additional prospective studies of sequential therapy using PEG IFN-α are needed as well.

Both HBsAg and HBcrAg levels at the time of NUC cessation were factors significantly associated with the response to NUC/IFN-α sequential therapy. HBsAg has been closely linked with PEG IFN-α therapy outcome.[24-27] Moucari *et al.*[26] analyzed HBeAg negative hepatitis B patients who had been treated with PEG IFN-α for 48 weeks and concluded that an early serum HBsAg drop was strongly predictive of a sustained virological response. Sonneveld *et al.*[24] assessed HBeAg positive hepatitis B patients who had received PEG IFN-α with or without LVD for 52 weeks and observed that patients who experienced no decline in HBsAg level from baseline at week 12 had little chance of achieving a sustained response and no possibility of HBsAg loss. HBcrAg includes antigens that are transcribed and translated from precore and core genes of the HBV genome, and HBeAg is a primary component of these antigens. Thus, our results were consistent with those described by Enomoto *et al.*[10] that the proportion of patients losing HBeAg positivity during ETV treatment was significantly higher in responders to ETV/IFN-α sequential therapy than in non-responders.

Hepatitis B surface antigen and HBcrAg levels have both been associated with intrahepatic HBV cccDNA, which is a key molecule in HBV replication whose value is closely related to HBV replication activity.[21,27,28] Several reports[27,29,30] have shown that HBV cccDNA level is associated with the response to antiviral therapy, such as with PEG IFN-α and NUC. Sung *et al.*[29] analyzed HBeAg positive hepatitis B patients who had been treated with either LVD monotherapy or a combination of PEG IFN-α and LVD and concluded that intrahepatic HBV cccDNA level at the end of therapy was superior to serum HBV DNA in predicting a sustained virological response. Serum HBV DNA is associated with intrahepatic HBV cccDNA and is widely used as a marker for HBV replication activity. However, such associations may be incompatible with antiviral therapies, and especially NUC treatment, because NUC directly hamper production of the HBV virion by inhibiting reverse transcription of pre-genomic RNA without affecting HBV cccDNA directly. As serum levels of HBsAg and HBcrAg are easier to measure than intrahepatic HBV cccDNA, these two antigen assays may be more suitable as surrogate markers for HBV replication activity in patients undergoing antiviral therapy. We previously reported that the combinational use of HBsAg and HBcrAg was beneficial to forecast the risk of hepatitis relapse after discontinuation of NUC.[13,14] The present study confirms this notion; it is possible that HBsAg and HBcrAg have complimentary roles in monitoring antiviral effects because the production of these two antigens is regulated by alternative enhancer–promoter systems in the HBV genome.

It is noteworthy that ROC analysis revealed maximal levels of ALT and HBV DNA to be closely associated with the 24-month response to NUC/IFN-α sequential therapy. We observed that patients with ALT higher than 128 IU/mL or HBV DNA higher than 6.0 log copies/mL during follow up were likely to be non-responders. When a relapse of hepatitis B was tentatively defined as ALT exceeding 128 IU/L during observation, relapses occurred frequently during the first 6 months after ceasing IFN-α and then became more sporadic afterwards. The timing of judgment of a virological response to NUC/IFN-α sequential therapy is critical when evaluating treatment efficacy. As this period is usually set at 6 months after completing therapy, our results confirm that 6 months is indeed appropriate. Our findings also suggest that maximal levels of ALT and HBV DNA are useful for monitoring the results of NUC/IFN-α sequential therapy. Accordingly, patients who are likely to be non-responders can now be identified as early as 24 weeks in advance and alternative strategies for treatment may be considered in a more timely fashion.

In conclusion, the combinational use of HBsAg and HBcrAg levels may be useful to predict the response to NUC/IFN-α sequential therapy. Maximal levels of ALT and HBV DNA during follow up may also be employed for monitoring the results of IFN-α sequential therapy.

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REFERENCES

1 Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. *Hepatology* 2007; **45**: 1056–75.

2 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
3 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733–45.
4 Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007; **132**: 1574–85.
5 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW. Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology* 2000; **32**: 635–9.
6 Honkoop P, de Man RA, Heijtink RA, Schalm SW. Hepatitis B reactivation after lamivudine. *Lancet* 1995; **346**: 1156–7.
7 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: results of a pilot study. *Hepatology* 2001; **34**: 573–7.
8 Shi M, Wang RS, Zhang H *et al.* Sequential treatment with lamivudine and interferon-alpha monotherapies in hepatitis B e antigen-negative Chinese patients and its suppression of lamivudine-resistant mutations. *J Antimicrob Chemother* 2006; **58**: 1031–5.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. A partially overlapping treatment course with lamivudine and interferon in hepatitis B e antigen-negative chronic hepatitis B. *Aliment Pharmacol Ther* 2006; **23**: 99–106.
10 Enomoto M, Nishiguchi S, Tamori A *et al.* Entecavir and interferon-alpha sequential therapy in Japanese patients with hepatitis B e antigen-positive chronic hepatitis B. *J Gastroenterol* 2013; **48**: 397–404.
11 Enomoto M, Tamori A, Nishiguchi S, Kawada N. Combination therapy with a nucleos(t)ide analogue and interferon for chronic hepatitis B: simultaneous or sequential. *J Gastroenterol* 2013; **48**: 999–1005.
12 Minami M, Okanoue T. Management of HBV infection in Japan. *Hepatol Res* 2007; **37**: S79–82.
13 Matsumoto A, Tanaka E, Suzuki Y *et al.* Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. *Hepatol Res* 2012; **42**: 139–49.
14 Tanaka E, Matsumoto A. Guidelines for avoiding risks resulting from discontinuation of nucleoside/nucleotide analogs in patients with chronic hepatitis B. *Hepatol Res* 2014; **44**: 1–8.
15 Orito E, Ichida T, Sakugawa H *et al.* Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590–4.
16 JSH Guidelines for the Management of Hepatitis B Virus Infection. *Hepatol Res* 2014; **44** (Suppl S1): 1–58.
17 Schuttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH. Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis B virus surface antigen (HBsAg). *J Clin Virol* 2010; **47**: 238–42.
18 Ronsin C, Pillet A, Bali C, Denoyel GA. Evaluation of the COBAS AmpliPrep-total nucleic acid isolation-COBAS TaqMan hepatitis B virus (HBV) quantitative test and comparison to the VERSANT HBV DNA 3.0 assay. *J Clin Microbiol* 2006; **44**: 1390–9.
19 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; **450**: 66–71.
20 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
21 Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27–33.
22 Kumada H, Okanoue T, Onji M *et al.* Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 1–7.
23 Cooksley WG, Piratvisuth T, Lee SD *et al.* Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. *J Viral Hepat* 2003; **10**: 298–305.
24 Sonneveld MJ, Rijckborst V, Boucher CA, Hansen BE, Janssen HL. Prediction of sustained response to peginterferon alfa-2b for hepatitis B e antigen-positive chronic hepatitis B using on-treatment hepatitis B surface antigen decline. *Hepatology* 2010; **52**: 1251–7.
25 Brunetto MR, Moriconi F, Bonino F *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2009; **49**: 1141–50.
26 Moucari R, Mackiewicz V, Lada O *et al.* Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients. *Hepatology* 2009; **49**: 1151–7.
27 Chan HL, Wong VW, Tse AM *et al.* Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462–8.
28 Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45**: 3942–7.
29 Sung JJ, Wong ML, Bowden S *et al.* Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. *Gastroenterology* 2005; **128**: 1890–7.
30 Wursthorn K, Lutgehetmann M, Dandri M *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 675–84.

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ORIGINAL ARTICLE

Retrospective Study

Mutations of pre-core and basal core promoter before and after hepatitis B e antigen seroconversion

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Abstract

AIM: To investigate the role of pre-core and basal core promoter (BCP) mutations before and after hepatitis B e antigen (HBeAg) seroconversion.

METHODS: The proportion of pre-core (G1896A) and basal core promoter (A1762T and G1764A) mutant viruses and serum levels of hepatitis B virus (HBV) DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen were analyzed in chronic hepatitis B patients before and after HBeAg seroconversion (n = 25), in those who were persistently HBeAg positive (n = 18), and in those who were persistently anti-HBe positive (n = 43). All patients were infected with HBV genotype C and were followed for a median of 9 years.

RESULTS: Although the pre-core mutant became predominant (24% to 65%, P = 0.022) in the HBeAg seroconversion group during follow-up, the proportion of the basal core promoter mutation did not change. Median HBV viral markers were significantly higher in patients without the mutations in an HBeAg positive status (HBV DNA: P = 0.003; HBsAg: P < 0.001; HB core-related antigen: P = 0.001). In contrast, HBV DNA (P = 0.012) and HBsAg (P = 0.041) levels were significantly higher in patients with the pre-core mutation in an anti-HBe positive status.

CONCLUSION: There is an opposite association of the pre-core mutation with viral load before and after HBeAg seroconversion in patients with HBV infection.

Key words: Seroconversion; Hepatitis B core-related antigen; Pre-core; Basal core promoter; Mutation; Hepatitis B surface antigen; Hepatitis B virus DNA

Core tip: The exact roles of pre-core (pre-C) and basal core promoter (BCP) mutations remain unclear before and after hepatitis B e antigen (HBeAg) seroconversion.

Here, although the pre-C mutant became predominant in the HBeAg seroconversion group during follow-up, the proportion of the BCP mutation did not change. Hepatitis B virus (HBV) viral markers were significantly higher in patients without the mutations in an HBeAg positive status. HBV DNA and hepatitis B surface antigen levels were higher in patients with the pre-C mutation in an anti-HBe positive status. Taken together, the association of the pre-C mutation on viral load appears to be opposite before and after HBeAg seroconversion in patients with HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma[1-4].

In the natural history of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and the remission of hepatitis[5-7]. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion[8,9].

Several mutations in the HBV genome have been reported to associate with HBeAg seroconversion. When the pre-core (pre-C) and core genes in the HBV genome are transcribed and translated in tandem, HBeAg is produced and secreted into the circulation[10,11]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of detectable HBeAg[12,13]. The double mutations of A1762T and G1764A in the basal core promoter (BCP) of the HBV genome have also been shown to reduce HBeAg synthesis by suppressing the transcription of pre-C mRNA[14-16]. However, the detailed mechanisms of HBeAg seroconversion, including the involvement of mutations that decrease the production of HBeAg, have not been fully clarified. Orito *et al*[17] reported that a predominance of the pre-C mutation was correlated with anti-HBe, while BCP mutations were not associated with either anti-HBe or HBeAg. We previously uncovered that the pre-C and BCP mutations were frequently seen in patients with active replication after HBeAg seroconversion, but not in those with inactive replication[18], which suggested that HBeAg seroconversion was not associated with either mutation in such patients. Since the follow-up duration of these previous reports was limited, this study analyzed the changes in pre-C and BCP mutations among patients who were followed over a longer time course. Furthermore, we assessed the mutations not only in patients who seroconverted from HBeAg to anti-HBe, but also in those whose HBeAg or anti-HBe positive status did not change during follow-up.

MATERIALS AND METHODS

Patients

Three groups of patients with chronic hepatitis B who were categorized according to HBeAg/anti-HBe positive status were enrolled between 1985 and 2000. The subjects were selected retrospectively from a database of patients who had been followed for at least two years, had not received anti-viral therapy, such as nucleos(t)ide analogues, and whose stored serum samples were available from both the start and end of follow-up. We recruited only patients with HBV genotype C since this genotype is predominant in Japan and because the clinical significance of pre-C and BCP mutations differs among genotypes. The first group consisted of 18 patients whose HBeAg was persistently positive throughout the study period. The second group contained 25 patients in whom HBeAg seroconverted to anti-HBe. The third group was made up of 43 patients whose anti-HBe was persistently positive.

Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions a minimum of 6 mo apart in all patients before the start of follow-up. Tests for hepatitis C and human immunodeficiency virus antibodies were negative in all subjects. Patients who demonstrated accompanying hepatocellular carcinoma or signs of hepatic failure at the initial follow-up were excluded from the study.

Stored serum samples were kept frozen at -20 ℃ or below until assayed. This study was approved by the Ethics Committee of Shinshu University School of Medicine.

Conventional hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and anti-HBe, were tested using commercially available enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan)[19]. HBsAg was quantified[20] using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 8.9 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal and no signal detection was considered to be a negative signal. Six HBV genotypes (A-F) were

Table 1 Clinical and virological backgrounds among 3 groups of patients classified according to changes in hepatitis B e antigen/anti-hepatitis B e

Characteristic	HBeAg/anti-HBe status			*P* value
	Continuously +/- (*n* = 18)	From +/- to -/+ (*n* = 25)	Continuously -/+ (*n* = 43)	
Age (yr)[1]	44 (24-63)	37 (18-53)	51 (25-77)	< 0.001
Gender (M:F)	11:7	14:11	24:19	> 0.2
Follow-up period (yr)[1]	6.3 (2.1-14.6)	10.8 (2.0-23.7)	8.5 (2.2-16.6)	0.006
Genotype C[2]	18 (100)	25 (100)	43 (100)	1
Viral markers at first follow-up				
HBV DNA (log copies/mL)[1]	8.6 (5.7-> 8.9)	6.1 (< 2.1-> 8.9)	< 2.1 (< 2.1-8.2)	< 0.001
HBsAg (log IU/mL)[1]	4.6 (1.6-5.5)	3.6 (-0.9-4.6)	2.6 (< 0.05-4.3)	< 0.001
HBcrAg (log U/mL)[1]	> 6.8 (5.5->6.8)	6.8 (3.1-> 6.8)	3.0 (< 3.0-6.8)	< 0.001
Viral markers at final follow-up				
HBV DNA (log copies/mL)[1]	7.1 (< 2.1-> 8.9)	3.3 (neg.-6.2)	< 2.1 (neg.-7.0)	< 0.001
HBsAg (log IU/mL)[1]	3.3 (1.0-5.1)	2.8 (< 0.05-2.8)	1.3 (< 0.05-4.2)	< 0.001
HBcrAg (log U/mL)[1]	6.7 (4.4-> 6.8)	< 3.0 (< 3.0-6.2)	< 3.0 (< 3.0-5.3)	< 0.001

[1]Data are expressed as the median (range); [2]Data are expressed as a positive number (%). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis B core-related antigen.

evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al*[22]. Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc.) as described previously[23,24]. The HBcrAg assay simultaneously measured all antigens (e, core, and p22cr) encoded by the pre-C/core genes of HBV. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL with a quantitative range of 3.0 to 6.8 log U/mL.

Determination of pre-C and BCP mutations

The pre-C and BCP mutations were determined using nucleic acid samples extracted from 100 μL of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in wild type HBV and A1896 in the mutant were determined by mini-sequence reactions using labeled nucleotides that were complementary to either the wild type or mutant[25]. The results were expressed as percent mutation rates according to the definition by Aritomi *et al*[26] Samples were judged as positive for the pre-C mutation when the mutation rate exceeded 50% in the present study since the mutation rate was found to steadily increase to 100% once surpassing 50%[25].

The double mutation in the BCP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories)[25,26]. This kit detected T1762 and/or A1764 using the polymerase chain reaction (PCR) with primers specific for either wild type or mutant BCP. Results were recorded as wild, mixed, or mutant type. The pre-C and BCP mutations were tested at the start and end of follow-up with kits having manufacturer-established detection limits of 1000 copies/mL.

Full HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously[27]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 3.1; Foster City, CA) with an automated ABI DNA sequencer (Model 3100, Applied Biosystems Carlsbad, CA).

Statistical analyses

The proportions of clinical factors were compared among groups using the χ^2 and Fisher's exact probability tests. Group medians were compared by means of the Mann-Whitney *U* test and Kruskal-Wallis test. The changes in proportions of the pre-C and BCP mutations between the study start and end points were compared using McNemar's test. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Patients

The clinical and virological backgrounds of the 3 groups are summarized in Table 1. Median age was lowest in patients with seroconversion, intermediate in those with persistent HBeAg, and highest in those with persistent anti-HBe. Gender ratio was similar among the 3 groups. Following our study design, all patients had HBV ge-