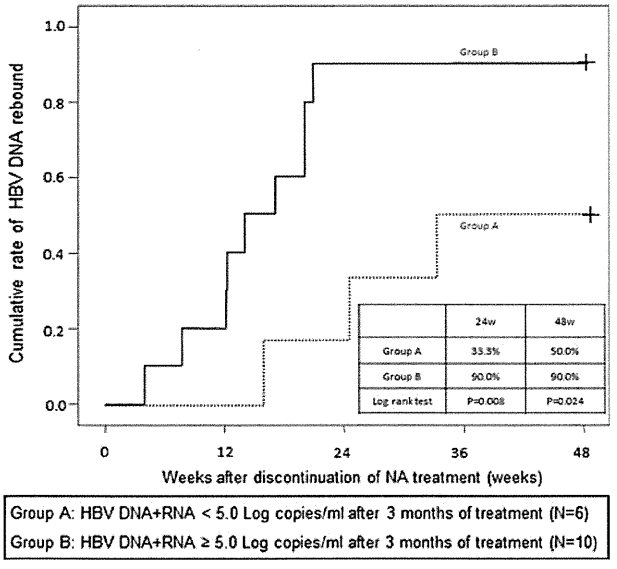

Fig. 5 Cumulative rate of ALT rebound after discontinuation of NA treatment in HBeAg-positive chronic hepatitis B patients. Six patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment were assigned to group A; the other ten patients, whose HBV DNA + RNA titers were ≥5.0 log copies/mL after 3 months of treatment, were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

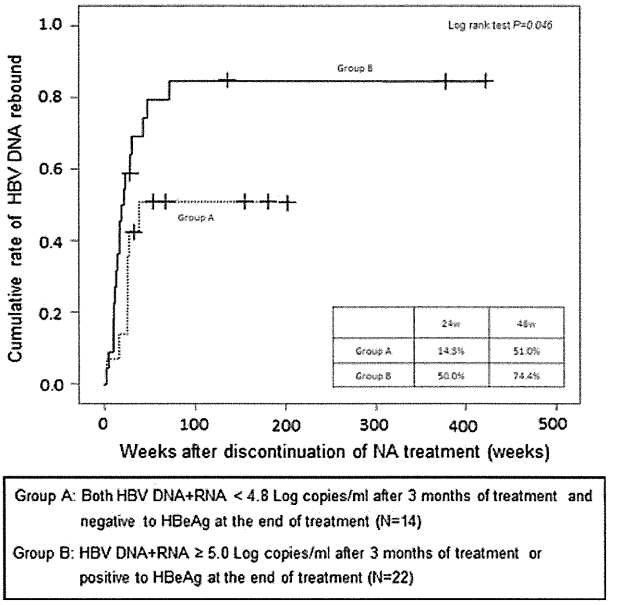

Fig. 6 Cumulative rate of ALT rebound after discontinuation of NA treatment by using combined criteria. The subjects were divided using combined criteria. Fourteen patients whose HBV DNA + RNA titers reached <5.0 log copies/mL after 3 months of treatment and who were HBeAg negative at the end of NA treatment were assigned to group A; the other 22 patients were assigned to group B. The cumulative ALT rebound rate in HBeAg-positive chronic hepatitis B patients was analyzed using the Kaplan–Meier method

DNA + RNA titer after 3 months of treatment was found to be significantly associated with HBV DNA rebound ($P = 0.043$, OR = 9.474; Table 2). Two other factors, HBV DNA titer after 3 months of treatment and HBsAg titer at the end of treatment, were marginally associated with HBV DNA rebound ($P = 0.074$, $P = 0.070$, respectively). After 3 months of NA treatment, HBV DNA titers decreased in both the HBV DNA relapse and non-relapse groups, but HBV DNA + RNA levels in the relapse group remained high. NA therapy suppressed the production of mature HBV particles in both groups, but in the HBV DNA relapse group, high HBV replication activity was likely maintained during the treatment, and immature HBV particles associated with HBV RNA genomes were continuously produced and accumulated in hepatocytes. After discontinuation of the treatment, these accumulated immature HBV particles may have been maturated and been released from the hepatocytes. Thus, rebound of HBV DNA titers occurred rapidly after the discontinuation of NA therapy.

Although the presence of HBeAg before treatment, HBV DNA and DNA + RNA titers after 3 months of treatment, and the presence of HBeAg, HBsAg titer, and HBV DNA + RNA titer at the end of treatment were all significantly associated with ALT rebound in the univariate analysis, only the presence of HBeAg at the end of treatment was identified as an independent predictive factor for ALT rebound following multivariate analysis (Table 4). HBeAg is commonly strongly associated with the activity of HBV replication, and HBV DNA levels are high in HBeAg-positive HBV carriers. Thus, HBe seroconversion usually indicates suppression of HBV activity, and the absence of HBeAg is thought to indicate the inactivation of HBV replication.

ALT rebound following the discontinuation of NA therapy was not observed in six of the 16 patients (37.5 %) who were HBeAg-positive at the end of treatment. After examining predictive factors for ALT rebound in these HBeAg-positive patients, only the HBV DNA + RNA titer after 3 months of treatment was identified as an independent predictive factor for ALT rebound in HBeAg-positive patients (Table 6). Although the presence of HBeAg indicates high activities of HBV replication and hepatitis, it is expected to be difficult to discontinue NA therapy without ALT rebound in these patients. However, these results indicate that HBV replication activities vary greatly among individuals and suggest that it might be possible to predict future replication activity based on HBV DNA + RNA titers after 3 months of treatment.

A limitation of this study is the small sample size; as such, selection bias might have affected the internal validity of the study. As it is not common to discontinue

NA therapy in Japan, we were only able to examine 36 subjects in our study. Because HBV-related markers such as HBsAg, HBcrAg, and HBV DNA + RNA titers varied widely among individuals, HBeAg an HBV DNA + RNA titers were only marginally associated with HBV DNA or ALT rebound after the discontinuation of NA therapy. In a previous study, Matsumoto et al. [34] analyzed predictive factors for the safe discontinuation of NA therapy in 126 clinical HBeAg-negative subjects from 12 clinical centers. These authors reported that HBsAg and HBcrAg titers at the end of treatment were predictive factors for the safe discontinuation of therapy. In our study, we also found that the absence of HBeAg at the end of treatment was important for the safe discontinuation of NA therapy, but we found no association between safety and HBsAg or HBcrAg titers. However, while HBsAg and HBcrAg are known to be associated with HBV replication activity, our results involving HBeAg and HBV DNA + RNA titers as important factors for safe discontinuation appear to be consistent.

In our study, the duration of NA therapy was quite short (mean duration was 36 weeks). Similar results might be observed if the NA therapy was extended, but it might be difficult to depress the potential of infected HBV replication with long-term NA therapy. HBsAg titers represent HBV replication in human hepatocytes, and it is difficult to decrease HBsAg levels by NA therapy. Thus, HBV DNA + RNA levels might be an important factor for predicting the HBV DNA or ALT rebounds.

As it may be difficult to discontinue therapy in patients with advanced liver fibrosis, our study subjects were selected based on liver spare capacities. As shown in Fig. 1, ALT rebound is likely to occur in most patients following the discontinuation of NA therapy, and severe hepatitis could occur in some patients. Thus, if the liver spare capacity were low, NA therapy would not be discontinued; the patients in this study were selected solely based on clinical aspects, which may have influenced our interpretation of the results.

In conclusion, HBV replication activity was found to be an important predictor of safe discontinuation of NA therapy. These findings suggest that monitoring of serum HBV DNA + RNA levels would be a useful method for predicting the re-activation of chronic hepatitis B following discontinuation of NA therapy.

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Conflict of interest None to declare.

References

1. Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med. 2004;350(11):1118–29.
2. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. Lancet. 1993;342(8883):1340–4.
3. Ohishi W, Fujiwara S, Cologne JB, Suzuki G, Akahoshi M, Nishi N, et al. Impact of radiation and hepatitis virus infection on risk of hepatocellular carcinoma. Hepatology. 2011;53(4):1237–45.
4. Brechot C, Gozuacik D, Murakami Y, Paterlini-Brechot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). Semin Cancer Biol. 2000;10(3):211–31.
5. Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Brechot C, et al. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. Gut. 2005;54(8):1162–8.
6. Nagaya T, Nakamura T, Tokino T, Tsurimoto T, Imai M, Mayumi T, et al. The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. Genes Dev. 1987;1(8):773–82.
7. Yaginuma K, Kobayashi H, Kobayashi M, Morishima T, Matsuyama K, Koike K. Multiple integration site of hepatitis B virus DNA in hepatocellular carcinoma and chronic active hepatitis tissues from children. J Virol. 1987;61(6):1808–13.
8. Conjeevaram HS, Lok AS. Management of chronic hepatitis B. J Hepatol. 2003;38[Suppl 1]:S90–103.
9. Kumada H, Okanoue T, Onji M, Moriwaki H, Izumi N, Tanaka E, 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):1–7.
10. Lee YS, Suh DJ, Lim YS, Jung SW, Kim KM, Lee HC, et al. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. Hepatology. 2006;43(6):1385–91.
11. Buti M, Jardi R, Cotrina M, Rodriguez-Frias F, Esteban R, Guardia J. Transient emergence of hepatitis B variants in a patient with chronic hepatitis B resistant to lamivudine. J Hepatol. 1998;28(3):510–3.
12. Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology. 1998;27(6):1711–6.
13. Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. Gastroenterology. 2007;132(4):1574–85.
14. Kobayashi M, Suzuki F, Akuta N, Yatsuji H, Hosaka T, Sezaki H, et al. Correlation of YMDD mutation and breakthrough hepatitis with hepatitis B virus DNA and serum ALT during lamivudine treatment. Hepatol Res. 2010;40(2):125–34.
15. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, et al. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology. 2007;46(1):254–65.
16. Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology. 2003;46(3):182–9.
17. Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, et al. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to lamivudine. Antimicrob Agents Chemother. 2004;48(9):3498–507.

18. Tenney DJ, Rose RE, Baldick CJ, Levine SM, Pokornowski KA, Walsh AW, et al. Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. Antimicrob Agents Chemother. 2007;51(3):902–11.
19. Yatsuji H, Hiraga N, Mori N, Hatakeyama T, Tsuge M, Imamura M, et al. Successful treatment of an entecavir-resistant hepatitis B virus variant. J Med Virol. 2007;79(12):1811–7.
20. Yatsuji H, Suzuki F, Sezaki H, Akuta N, Suzuki Y, Kawamura Y, et al. Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. J Hepatol. 2008;48(6):923–31.
21. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. Gastroenterology. 2009;137(5):1593–608. e1–2.
22. Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. Hepatology. 2007;45(5):1179–86.
23. Huang YW, Chayama K, Tsuge M, Takahashi S, Hatakeyama T, Abe H, et al. Differential effects of interferon and lamivudine on serum HBV RNA inhibition in patients with chronic hepatitis B. Antivir Ther. 2010;15(2):177–84.
24. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection: representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res. 2001;7(7):2005–15
25. Zhang W, Hacker HJ, Tokus M, Bock T, Schroder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. J Med Virol. 2003;71(1):24–30.
26. Pugh JC, Bassendine MF. Molecular biology of hepadnavirus replication. Br Med Bull. 1990;46(2):329–53.
27. Loguercio C, Di Pierro M, Di Marino MP, Federico A, Disalvo D, Crafa E, et al. Drinking habits of subjects with hepatitis C virus-related chronic liver disease: prevalence and effect on clinical, virological and pathological aspects. Alcohol Alcohol. 2000;35(3):296–301.
28. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol. 2002;40(2):439–45.
29. 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(1):27–33.
30. Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med. 2006;354(10):1001–10.
31. Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, et al. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med. 1998;339(2):61–8.
32. Lai CL, Rosmawati M, Lao J, Van Vlierberghe H, Anderson FH, Thomas N, et al. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. Gastroenterology. 2002;123(6):1831–8.
33. Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z, et al. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. N Engl J Med. 2006;354(10):1011–20.
34. Matsumoto A, Tanaka E, Suzuki Y, Kobayashi M, Tanaka Y, Shinkai N, 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(2):139–49.

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総　説

肝疾患の疾患関連遺伝子
—GWASを含めた最近の知見から—

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要旨：ゲノムワイド関連解析（GWAS）の登場以降，肝疾患領域においてもさまざまな疾患病態についての遺伝子多型解析が急速に拡大してきた．その結果*IL28B*のように予測マーカーとして臨床応用が急速に進展したSNPがある一方で，進捗のないものも多い．また発見された全SNPでもその遺伝性のごく一部分しか説明し得ない事例が多数報告されており，GWASの改良のみならずrare SNPや構造多型，遺伝子間作用，遺伝子環境間作用，epigeneticsなどへのアプローチの必要性が提唱されている．本総説では主にGWASによる肝疾患関連遺伝子研究について概括するとともに，疾患関連遺伝子研究の問題点と方向性についても論じる．

索引用語：疾患関連遺伝子，遺伝子多型，SNP，GWAS

はじめに

癌や生活習慣病など多くの疾患は，その発症や進展に複数の環境要因と遺伝要因がさまざまな割合で関与する，多因子性疾患と考えられている．ゲノムワイド関連解析（genome-wide association study；GWAS）の登場によって，これら多因子性疾患の遺伝要因についての研究が急速に拡大したが，問題点や限界も指摘されてきている．本稿では主にGWASによって同定された肝疾患関連遺伝子について概括し，続いて疾患関連遺伝子研究の問題点と方向性についても論じることとする．

I　GWASとは

GWASは，ある疾患に関与する遺伝要因のゲノム上の位置を網羅的に調べる方法の1つである．2005年にタイピングアレイを用いた最初のGWAS論文が発表されて以降，これまでに約1600の論文と約1万のsingle nucleotide polymorphism（SNP）が報告されている（www.genome.gov/gwastudies/）．遺伝的要因の探索に際して連鎖解析と比較すると，関連解析は個々の効果が小さい多因子性疾患においてより有用で，検出力が高く，より狭い範囲に遺伝子座（locus）を絞り込むことができる[1)]．そのため全ゲノムをカバーするためには非常に多数のマーカーが必要で，マーカー数の増大にともない検定の有意水準が非常に小さくなる．十分な検出力を得るためには必要サンプル数も増大するが，血縁関係のない

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Recent progress in the study of genetic variants associated with liver diseases

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Table 1. PBC, PSC に関する GWAS

著者	Year [ref]	Ethnicity	解析サンプル数		
			Case (Validation)	Control (Validation)	全体
Hirschfield	2009 [4]	European-American, European-Canadian	505 (526)	1507 (1206)	3744
Hirschfield	2010 [5]	European-American	857 (combined with previous GWAS)	3198	4055
Liu	2010 [6]	European-Canadian, Italian	453 (481)	945 (3706)	5585
Mells	2011 [7]	European	2774 (620)	9814 (2514)	15722
Nakamura	2012 [8]	Japanese	487 (787)	476 (615)	2365
Karlsen	2010 [9]	European	285 (766)	298 (2935)	4284
Melum	2010 [10]	European	715 (1025)	2962 (1837)	6539
Folseraas	2012 [11]	European, European-American	715 (1221)	2962 (3508)	8406

表現型	Location	候補遺伝子	SNP ID	オッズ比（95%CI）またはβ（SE）	備考
PBC	6p21.3	*HLA-DQB1*	rs2856683	1.75（1.55～1.98）	
PBC	3q25.33	*IL12A*	rs6441286	1.54（1.38～1.72）	
PBC	1p31.3	*IL12RB2*	rs3790567	1.51（1.33～1.70）	
PBC	7q32.1	*IRF5-TNPO3*	rs10488631	1.57（1.38～1.77）	[4] とのメタアナリシス
PBC	17q12-21	*ZPBP2*	rs11557467	0.72（0.66～0.79）	
PBC	1p36	*MMEL1*	rs3748816	1.33（1.20～1.17）	
PBC	19q13.33	*SPIB*	rs3745516	1.46	
PBC	7q32.1	*IRF5-TNPO3*	rs10488631	1.63	
PBC	17q12	*IKZF3*	rs9303277	1.38	
PBC	6p21.32	*HLA-DQB1*	rs7774434	1.75	replication
PBC	3q25.33	*IL12A*	rs6441286	1.44	replication
PBC	1p31.3	*IL12RB2*	rs3790567	1.48	replication
PBC	2q32.2	*STAT4*	rs10931468	1.5（1.37～1.64）	
PBC	1q31.3	*DENND1B*	rs12134279	1.34（1.25～1.45）	
PBC	11q23.3	*CXCR5*	rs6421571	1.37（1.25～1.50）	
PBC	16p13.13	*CLEC16A*	rs12924729	1.29（1.20～1.38）	
PBC	4q24	*NFKB1*	rs7665090	1.26（1.18～1.34）	
PBC	5p13.2	*IL7R*	rs860413	1.3（1.21～1.40）	
PBC	14q24.1	*RAD51L1*	rs911263	1.29（1.20～1.39）	
PBC	3q13.33	*CD80*	rs2293370	1.35（1.23～1.47）	
PBC	16q24.1	Intergenic	rs11117432	1.31（1.21～1.43）	
PBC	22q13.1	*MAP3K7IP1*	rs968451	1.27（1.18～1.38）	
PBC	12p13.31	*TNFRSF1A*	rs1800693	1.22（1.14～1.30）	
PBC	7p14.1	Intergenic	rs6974491	1.25（1.16～1.36）	
PBC	3p24.3	*PLCL2*	rs1372072	1.2（1.12～1.27）	[6] とのメタアナリシス
PBC	14q32.32	*TNFAIP2*	rs8017161	1.22（1.16～1.27）	
PBC	11q13.1	*RPS6KA4*	rs538147	1.23（1.15～1.31）	
PBC	6p21.32	*HLA-DQB1*	rs7774434	1.6（1.48～1.73）	replication
PBC	1p31.3	*IL12RB2*	rs17129789	1.52（1.39～1.67）	replication
PBC	7q32.1	*IRF5*	rs12531711	1.58（1.41～1.76）	replication
PBC	3q25.33	*IL12A*	rs485499	1.38（1.28～1.50）	replication
PBC	19q13.33	*SPIB*	rs3745516	1.38（1.32～1.44）	replication
PBC	17q12	*ORMDL3*	rs7208487	1.32（1.18～1.48）	replication
PBC	1p36	*MMEL1*	rs10752747	1.13（1.04～1.22）	replication
PBC	9q32	*TNFSF15*	rs4979462	1.56（1.39～1.76）	
PBC	11q23.1	*POU2AF1*	rs4938534	1.39（1.24～1.56）	
PBC	4q23	*NFKB1*	rs7665590	1.35（1.21～1.52）	replication
PBC	3q13.33	*CD80*	rs2293370	1.48（1.29～1.68）	replication
PBC	5p13.2	*IL7R*	rs6890853	1.47（1.28～1.69）	replication
PBC	17q12	*IKZF3*	rs9303277	1.44（1.28～1.63）	replication
PBC	2q32.3	*STAT4*	rs7574865	1.35（1.19～1.52）	replication
PSC	6p21	*HLA-B*	rs3099844	4.8（3.8～6.5）	
PSC	3p21.31	*MST1*	rs3197999	1.39（1.24～1.56）	
PSC	2q13	*BCL2L11*	rs6720394	1.29（1.10～1.51）	
PSC	1p36	*MMEL1-TNFRSF14*	rs3748816	0.79	

個人の集団を解析対象とするため連鎖解析と比較して大規模な集団を集めやすいという特徴がある．この解析手法の基礎理論は1996年には発表されていたが[2]，その後国際HapMapプロジェクトによるヒトハプロタイプ地図の完成や，高密度SNPタイピングアレイ技術などの革新によって，ついにGWASによる疾患関連遺伝子解析が実現した．以下に肝臓疾患領域におけるこれまでのGWASの状況について概説する．

II 自己免疫性肝炎（autoimmune hepatitis；AIH）

AIHについてはSNPベースのGWASによる疾患関連遺伝子の同定はいまだ報告されていないが，Yokosawaらは81人の1型AIH症例を用いて400個のmicrosatelliteによるゲノムワイドな関連解析を行ったところ，18p11.22（p＝0.008）と11p15.1（p＝0.013）の2つの遺伝子座が有意にAIHと関連していた[3]．

III 原発性胆汁性肝硬変（primary biliary cirrhosis；PBC）

Hirschfieldらは，アメリカおよびカナダ人のPBC 1031人と健常人2713人を用いたGWASによって，PBCと有意に関連する*HLA-DQB1*，*IL12A*，*IL12RB2*遺伝子領域のSNPを発見し[4]，後にコホートを追加したメタアナリシス解析でさらに3つの遺伝子座（*IRF5-TNPO3*，*ZPBP2*，*MMEL1*）を同定している[5]．Liuらは，イタリア人とカナダ人のPBC 934人とコントロール4187人による解析から，PBCと関連するSNPとしてHirschfieldらの報告した3遺伝子座に加えて*SPIB*，*IRF5-TNPO3*，*IKZF3*の遺伝子座のSNPを発見した[6]．Mellsらは，ヨーロッパ人のPBC 3394人と健常人12328人を用いて，PBCと関連する既報のGWASの5遺伝子座に加え*STAT4*，*CD80*，*IL7R*，*CXCR5*，*TNFRSF1A*，*NFKB1*など12遺伝子領域のSNPを発見した[7]．Nakamuraらは，日本人のPBC 1274人と健常人1091人の解析からPBCと関連する*TNFSF15*と*POU2AF1*遺伝子のSNPを発見し，既報の遺伝子座においても有意な（*IL7R*，*IKZF3*，*CD80*）または示唆的な（*STAT4*，*NFKB1*）関連性を認めた[8]（Table 1）．

IV 原発性硬化性胆管炎（primary sclerosing cholangitis；PSC）

Karlsenらは，ヨーロッパ人のPSC 1051人とコントロール3233人を用いて，PSCと関連するHLA-B領域のSNPを発見した[9]．Melumらは，ヨーロッパ系のPSC 1740人とコントロール4799人を用いて，PSC発症と関連する*MST1*と*BCL2L11*の遺伝子多型を発見した[10]．Folseraasらは，MelumらのGWASデータにimputingによる関連解析を行い，*MMEL1-TNFRSF14*遺伝子座のSNPがPSCと関連することを発見した．この遺伝子座はceliac disease，multiple sclerosis，PBCの候補遺伝子としても報告されている[11]（Table 1）．

V 非アルコール性脂肪肝・肝硬変（non-alcoholic fatty liver disease；NAFLD・non-alcoholic steatohepatitis；NASH）

Romeoらは，アフリカ系1032人，ヨーロッパ系696人，ヒスパニック系383人を用いて，9229個のnonsynonymous SNPからNAFLDと関連する*PNPLA3*遺伝子領域の2つのSNP，rs738409（I148M），rs6006460（S453I）を発見した．これらは互いに独立でrs6006460はヨーロッパ系，ヒスパニック系ではまれな多型であった．rs738409は血清ALT値との関連性も認めている[12]．Yuanらはヨーロッパ系の3つのコホート計12419人を用いて，肝機能検査と関連する遺伝子多型をGWAS解析したところ，血清ALT値と*PNPLA3*遺伝子座のrs2281135が有意に相関することを発見した[13]．Chalasaniらはヨーロッパ人のNAFLD女性236人を用いて，NAFLD activity scoreと関連する*FDFT1*遺伝子領域のSNPをはじめとして5つのSNPにおいて，NAFLDに関する表現型と有意な関連性を認めた[14]．Speliotesらは，ヨーロッパ人7176人のCT上の肝脂肪化度についてのGWASデータのimputingによる関連解析と，それに続くNAFLD 592人とコントロール1405人によるreplicationによって，NAFLDと関連する*PNPLA3*，*NCAN*遺伝子領域のSNPを同定した[15]．Kawaguchiらは日本人のNAFLD

529人とコントロール932人を用いて，*PNPLA3*遺伝子座のrs738409において有意な関連性を認め，層別化による検討ではMatteoniらの分類type 4のみで関連性が認められた[16]．Adamsらはヨーロッパ人のNAFLD 126人とコントロール802人を用いたGWASで，10^{-5}未満のP値のSNPのなかで*GC*（vitamin D-binding protein）および*LCP1*の肝臓におけるmRNA発現量についてNASH群（n=8）とコントロール群（n=5）間で有意差を認めた．また，血清中のビタミンD結合タンパク質の量とsteatosis gradeの間には負の相関が認められた[17]．Kitamotoらは日本人のNAFLD 1326人とコントロール1184人の解析から，*PNPLA3*および近隣の*SAMM50*，*PARVB*遺伝子座のSNPがそれぞれ独立してNAFLDや肝脂肪化，線維化に関連していることを示した[18]（Table 2）．

VI 胆石症・ビリルビン代謝

Buchらはドイツ人4078人とチリ人334人のGWASから，胆石症に関連する遺伝子多型を*ABCG8*遺伝子領域に発見した[19]．Johnsonらは血液生化学検査の3件のGWAS研究のメタアナリシスによって，*UGT1A1*，*SLCO1B1*，*LST-3TM12*，*SLCO1A2*のSNPが血清総ビリルビンと関連することを報告した[20]．これらのSNPについてBuchらは，胆石症との関連解析および胆石組成との関連解析を通じて*UGT1A1*のSNPが特に男性において胆石症と関連することを示した[21]（Table 2）．

VII B型慢性肝炎

Kamataniらは，日本人とタイ人のB型慢性肝炎（chronic hepatitis B；CHB）2086人とコントロール4301人のGWASによって，*HLA-DPA1*と*HLA-DPB1*領域にhepatitis B virus（HBV）持続感染と関連するSNPを発見し，さらにハプロタイプ解析によってリスクハプロタイプ（*HLA-DPA1*0202-DPB1*0501*および*HLA-DPA1*0202-DPB1*0301*）と，protectiveハプロタイプ（*HLA-DPA1*0103-DPB1*0402*および*HLA-DPA1*0103-DPB1*0401*）を同定した[22]．Mbarekらは日本人のCHB 2667人，コントロール6496人によるGWASの結果，Kamataniらの報告した2個のSNPの関連性の再現性確認と，また新たに*HLA-DQB1*（rs2856718）と*HLA-DQB2*（rs7453920）の2個のSNPがCHBと関連することを見出した[23]．Liuらは中国人の無症候性HBVキャリアー1944人とCHB 854人の解析から，*GRIN2A*遺伝子座のrs11866328が肝炎の顕性化に関連することを報告した[24]．Pngらはインドネシア人のHBワクチン接種者3614人のGWASによって，HBワクチン接種後の抗体値に関連するSNPを*HLA-DR*，*HLA class III*，*HLA-DPB1*に認め，*HLA-DPB1*についてはKamataniらの報告と同一のSNPであった[25]．Nishidaらは，日本人および韓国人1793人（CHB 781人，健常人571人，HBV感染自然治癒441人）のGWASから，Kamataniらの報告した*HLA-DBA1*，*HLA-DPB1*領域の2個のSNPが自然治癒にも関連することを報告した[26]（Table 3）．

VIII 肝硬変・線維化

Patinらはヨーロッパ人のC型慢性肝炎（chronic hepatitis C；CHC）2123人を用いて，肝線維化と関連する2つの遺伝子多型を発見した[27]．Urabeらは，日本人のhepatitis C virus（HCV）による肝硬変1618人と慢性肝炎4854人の解析から*HLA class II*領域の2つのSNPと，さらには*HLA-DQA1*0601*が独立して肝硬変に関連することを報告した[28]（Table 4）．

IX 肝 癌

Zhangらは中国人のHBVによる肝癌2310人とCHB 1789人のGWASの結果，*KIF1B*のSNP rs17401966がHBVに起因する肝癌発症に関連することを報告した[29]．Cliffordらは，東アジア人のHBVまたはHCVによる肝癌386人とコントロール587人のGWASでは有意なSNPを認めなかったが，対肝硬変86人との比較において*TPTE2*および*DDX18*遺伝子座のSNPがHBVに起因する肝癌発症に関連することを報告した[30]．Chanらは中国人のHBVによる肝癌595人とCHB 825人の解析から，特に有意な遺伝子座を発見しなかったことを報告した[31]．Kumarらは日本人のHCVによる肝癌1394人とコント

Table 2. NAFLD, NASH, 胆石症, ビリルビン代謝に関するGWAS

著者	Year [ref]	Ethnicity	解析サンプル数 Case (Validation)	Control (Validation)	全体
Romeo	2008 [12]	696 European American, 1032 African American, 383 Hispanics			2111
Yuan	2008 [13]	European	7715 (4704)		12419
Chalasani	2010 [14]	Caucasian (women)	236		236
Speliotes	2011 [15]	European	7176 (CT hepatic steatosis) (NAFLD 592)	(control 1405)	9173
Kawaguchi	2012 [16]	Japanese	529	932	1461
Adams	2013 [17]	European	126	802	928
Kitamoto	2013 [18]	Japanese	392 (172)	934 (1012)	2510
Buch	2007 [19]	German, Chilean	280 (2000)	360 (1772)	4412
Johnson	2009 [20]	3 cohorts (FHS, RS, AGES-Reykjavik)	3424+3847+2193		9464

Table 3. HBV感染に関するGWAS

著者	Year [ref]	Ethnicity	解析サンプル数 Case (Validation)	Control (Validation)	全体
Kamatani	2009 [22]	Japanese	179 [616]	934 [1267]	6387
		Japanese, Thai	(991+308)	(1553+546)	
Mbarek	2011 [23]	Japanese	458 (2209)	2056 (4440)	9163
Liu	2011 [24]	Chinese	1729 (215)	628 (226)	2798
Png	2011 [25]	Indonesian	1683 (1931)		3614
Nishida	2012 [26]	Japanese	181	184 [185]	
		Japanese, Korean	(256+344)	(236+151) [150+106]	

表現型	Location	候補遺伝子	SNP ID	オッズ比（95%CI）またはβ（SE）	備考
hepatic fat levels	22q13	*PNPLA3*（I148M）	rs738409		9229 SNPs
lower hepatic fat content	22q13	*PNPLA3*（S453I）	rs6006460		
ALT level	22q13	*PNPLA3*	rs2281135	0.060（0.007）	
NAFLD activity score	8p23	*FDFT1*	rs2645424	−0.76	
degree of fibrosis	7p14	Intergenic	rs343064	1.29	
lobular inflammation	10q22	*COL13A1*	rs1227756	0.58	
lobular inflammation	11q13	*LTBP3*	rs6591182	0.54	
lobular inflammation	12p13	*EFCAB4B*	rs887304	0.37	
AST level	4q32.3	*DDX60L-PALLD*	rs2710833	0.39	
NAFLD	22q13	*PNPLA3*	rs738409	3.26（2.11 ～ 7.21）	replication
NAFLD	19p12	*NCAN*	rs2228603	1.65（1.15 ～ 2.87）	
NAFLD	22q13	*PNPLA3*	rs738409	1.66（1.43 ～ 1.94）	replication
NAFLD	4q12	*GC*	rs222054	2.54	
NAFLD	13q14	*LCP1*	rs7324845	3.29	
NAFLD	22q13	*PNPLA3*	rs738409	2.05	replication
NAFLD	22q13	*SAMM50*	rs738491	1.79（1.41 ～ 2.27）	
NAFLD	22q13	*PARVB*	rs6006473	1.74（1.38 ～ 2.21）	
gallstone	2p21	*ABCG8*	rs11887534	2.2（1.80 ～ 2.60）	D19H
total serum bilirubin	2q37	*UGT1A1*	rs6742078	0.239（0.009）	
total serum bilirubin	12p	*SLCO1B1*	rs4149056	0.05（0.01）	V174A
total serum bilirubin	12p12.2	*LST-3TM12*	rs2417873	0.05（0.01）	
total serum bilirubin	12p12	*SLCO1A2*	rs4149000	0.06（0.01）	

表現型	Location	候補遺伝子	SNP ID	オッズ比（95%CI）またはβ（SE）	備考
CHB	6p21	*HLA-DPA1*	rs3077	0.57（0.49 ～ 0.66）	[GWAS 2nd]
CHB	6p21	*HLA-DPB1*	rs9277535	1.75（1.61 ～ 1.92）	
CHB	6p21.32	*HLA-DQB1*	rs2856718	1.56（1.45 ～ 1.67）	
CHB	6p21.32	*HAL-DQB2*	rs7453920	1.81（1.62 ～ 2.01）	
CHB	6p21.32	*HLA-DPA1*	rs3077	1.87（1.73 ～ 2.01）	replication
CHB	6p21.32	*HLA-DPB1*	rs9277535	1.77（1.65 ～ 1.91）	replication
CHB vs ASC	16p13	*GRIN2A*	rs11866328	1.68（1.40 ～ 2.02）	
HB vaccine response	6p21.32	*HLA-DR*	rs3135363	1.53（1.35 ～ 1.74）	
HB vaccine response	6p21.33	*HLA*	rs9267665	2.05（1.64 ～ 2.57）	
HB vaccine response	6p21.32	*HLA-DPB1*	rs9277535	1.39（1.23 ～ 1.59）	
CHB vs control	6p21	*HLA-DPA1*	rs3077	0.42（0.30 ～ 0.58）	replication
CHB vs control	6p21	*HLA-DPB1*	rs9277542	0.42（0.31 ～ 0.58）	replication
CHB vs [resolver]	6p21	*HLA-DPA1*	rs3077	0.44（0.32 ～ 0.61）	
CHB vs [resolver]	6p21	*HLA-DPB1*	rs9277542	0.51（0.37 ～ 0.70）	

Table 4. 肝硬変，HCC に関する GWAS

著者	Year [ref]	Ethnicity	解析サンプル数			表現型
			Case (Validation)	Control (Validation)	全体	
Patin	2012 [27]	European	1161 (962)		CHC 2123	F0 ～ 4 Duration F0 ～ 1/F3 ～ 4
Urabe	2013 [28]	Japanese	LC 682 (936)	CHC 1045 (3809)	6472	LC vs CHC
Zhang	2010 [29]	Chinese	348 (1962)	359 (1430)	4099	HCC vs CHB
Clifford	2010 [30]	Korean, Chinese	HCC 180 (206) LC 66 (20)	Control 271 (316)	973	HCC vs Control HCC vs LC HCC vs LC
Chan	2011 [31]	Chinese	95 (500)	97 (728)	1420	HCC vs CHB
Kumar	2011 [32]	Japanese	721 (673)	2890 (2596)	6880	HCC vs Control
Miki	2011 [33]	Japanese	212 (710)	765 (1625)	3312	HCC vs CHC
Li	2012 [34]	Chinese	1538 (4431)	1465 (4725)	12159	HCC vs CHB HCC vs CHB
Jiang	2013 [35]	Chinese	1161 (4319)	1353 (4966)	11799	HCC vs CHB HCC vs CHB

Table 5. 薬剤応答性，薬剤性肝障害に関する GWAS

著者	Year [ref]	Ethnicity	解析サンプル数		
			Case (Validation)	Control (Validation)	全体
Ge	2009 [36]	European (African, Hispanics)	SVR 488 (45＋38)	NVR 383 (146＋37)	1137
Tanaka	2009 [37]	Japanese	72 (122)	82 (50)	326
Suppiah	2009 [38]	European	131 (261)	162 (294)	848
Fellay	2010 [45]	European-American African-American, Hispanics	988 (198＋100)		CHC 1286
Ochi	2010 [46]	Japanese	453 (470)		CHC 923
Tanaka	2011 [48]	Japanese	303 (391)		CHC 694
Thompson	2011 [49]	European-American African-American, Hispanics	984 (201＋99)		CHC 1284
Daly	2009 [50]	European	51 (64)	282	333
Singer	2010 [51]	Caucasians, Hispanics	139 (98)	481 (405)	1123
Lucena	2011 [52]	European	201	532	

Location	候補遺伝子	SNP ID	オッズ比（95%CI）またはβ（SE）	備考
3q23	*RNF7*	rs16851720	−0.23（0.31 ～ 0.15）（AC to AA） −0.46（0.54 ～ 0.38）（CC to AA）	linear regression (in all)
2q14	*MERTK*	rs4374383	0.19（0.10 ～ 0.37）	Cox proportional (in blood transfusion)
6p21	*C6orf10*	rs910049	1.46（1.28 ～ 1.62）	
6p21	*BTNL2-HLA-DRA*	rs3135363	1.37（1.24 ～ 1.51）	
1p36	*KIF1B*	rs17401966	1.64（1.49 ～ 1.82）	
—	—	—	—	
13q12	*TPTE2*	rs2880301	0.273（0.191 ～ 0.390）	
2q14	*DDX18*	rs2551677	3.383（2.070 ～ 5.529）	
—	—	—	—	
6p21.33	*MICA*	rs2596542	1.39（1.27 ～ 1.52）	
22q12.2	*DEPDC5*	rs1012068	1.75（1.51 ～ 2.03）	
6p21.32	*HLA-DQA1-HLA-DRB1*	rs9272105	1.28（1.22 ～ 1.35）	
21q21.3	*GRIK1*	rs455804	1.19（1.12 ～ 1.25）	
6p21.32	*HLA-DQ*	rs9275319	1.49（1.36 ～ 1.63）	
2q32.3	*STAT4*	rs7574865	1.21（1.14 ～ 1.28）	

表現型	Location	候補遺伝子	SNP ID	オッズ比（95%CI）またはβ（SE）	備考
SVR vs NVR	19q13.2	*IL28B*	rs12979860	2.0（1.8 ～ 2.3）（CC to TT）	
SVR vs NVR	19q13.2	*IL28B*	rs8099917	27.2（13.9 ～ 53.4）	
SVR vs NVR	19q13.2	*IL28B*	rs8099917	1.98（1.57 ～ 2.52）	replication
ribavirin induced anemia	20p13	*ITPA*	rs7270101		
	20p13	*ITPA*	rs1127354		
ribavirin induced anemia	20p13	*ITPA*	rs1127354	0.44	replication
change of Hb	20p13	*ITPA*	rs6139030	25（11.11 ～ 50.0）	
change of Hb	20p13	*DDRGK1*	rs11697186	33.33（12.50 ～ 100）	
decrease of PLT	20p13	*DDRGK1*	rs11697186	4.5（3.10 ～ 6.50）	
decrease of PLT	20p13	*ITPA*	rs6139030	3.9（2.80 ～ 5.50）	
IFN-related cytopenia	20p13	*ITPA*	rs965469		
flucloxacillin DILI	6p21.3	*HCP5*	rs2395029		
flucloxacillin DILI	3q27	*ST6GAL1*	rs10937275		
lumiracoxib DILI	6p21.3	*C6orf10*	rs3129900	3.5（2.6 ～ 4.8）	
amoxicillin DILI	6p21.33	*HCP5, HLA-B*	rs2395029	45（19.4 ～ 105）	replication
amoxicillin DILI	3q27.3	*ST6GAL1*	rs10937275	4.1	replication

ロール5486人の解析から，HBVに起因する肝癌発症に関連する*MICA*遺伝子座上のSNPを発見した．またMICAタンパクの血中濃度はリスクアレル保有者で有意に低いことが判明した[32]．Mikiらは日本人のHCVによる肝癌922人と非肝癌2390人を用いたGWASによって，HCVに起因する肝癌発症と関連する*DEPDC5*領域のSNPを発見した[33]．Liらは中国人のHBVによる肝癌5969人とCHB 6190人の解析から*HLA-DRB1-DQA1*領域と*GRIK1*のSNPがHBVに起因する肝癌発症に関連することを報告した[34]．Jiangらは中国人のHBVによる肝癌5480人と非肝癌6319人を用いたGWASによって，HBVに起因する肝癌発症と関連する2つのSNPを*HLA-DQ*および*STAT4*遺伝子座に発見した[35]（Table 4）．

X 薬剤応答性

1. CHCのペグインターフェロン・リバビリン治療応答性

Geらは，ヨーロッパ系アメリカ人，アフリカ系アメリカ人，ヒスパニック系アメリカ人のペグインターフェロン・リバビリン併用療法を実施したCHC（1型）症例1137人によるGWASによって，*IL28B*遺伝子領域のSNPが治療応答性に関連することを発見した[36]．Tanakaらも日本人のCHC（1型）症例326人によるGWASで，同じく*IL28B*遺伝子座のSNPとペグインターフェロン・リバビリン併用療法の治療効果の間に有意な関連性を報告した[37]．Suppiahらは6つのヨーロッパ系コホート合計848人のペグインターフェロン・リバビリン併用療法を受けたCHC症例によるGWASの結果，*IL28B*遺伝子座のSNPについて同様の関連性を発見している[38]（Table 5）．このSNPの効果はHCVサブタイプ2型やインターフェロン単独療法においても認められている[39][40]．また，HCVコア領域のアミノ酸変異[41]，肝臓のインターフェロン誘導遺伝子の発現量[42]や脂肪化[43]とも相関を認め，HCVの変異と宿主応答を考えるうえで興味深い．この遺伝子座については周辺リシーケンスによるファインマッピング[40]や，各種機能解析による遺伝子発現調節機構の解明が試みられているが，最近*IL28B*近傍に*Interferon λ4*遺伝子が発見され，既報のSNPと連鎖したフレームシフト多型によって*Interferon λ4*遺伝子の発現が調節されることが報告された[44]．

2. リバビリン誘導性貧血

Falleyらは，ヨーロッパ系，アフリカ系，ヒスパニック系アメリカ人計1286人のペグインターフェロン・リバビリン2剤併用療法を施行したCHC症例のGWASによって*ITPA*遺伝子領域の2つのSNP（rs7270101，rs1127354）がリバビリン誘導性貧血に関連することを報告した[45]．Ochiらは2剤併用療法を行った923人のCHC症例のGWASによって，日本人においては*ITPA*遺伝子上のnonsynonymousな1個のSNP（rs1127354）のみがリバビリン誘導性貧血に関連していることを報告した[46]（Table 5）．2剤併用療法およびテラプレビルを加えた3剤併用療法のいずれにおいても，*ITPA*遺伝子多型は治療中のリバビリンの減量に影響するが，治療効果までの影響はなかったとする報告が多い[45][47]．

3. インターフェロンによる血球減少

Tanakaらはペグインターフェロン・リバビリン療法を受けたCHC症例694人のGWAS解析により，インターフェロンによる血小板減少に有意に関連するSNPsを*DDRGK1-ITPA*遺伝子領域に発見した．同時に行ったリバビリン誘導性貧血についてのGWASでも同じSNPにおいて強い関連性を認め，いずれのSNPも*ITPA*の機能性SNP（rs1127354）と強い連鎖不平衡状態であった．リバビリンによるヘモグロビン減少が大きいほど反応性血小板増加は大きく，反応性血小板産生増加に起因すると考えられた[48]．Thompsonらもペグインターフェロン・リバビリン療法を受けたCHC症例1284人のGWAS解析により，インターフェロンによる血小板減少に有意に関連するSNPsを*ITPA*遺伝子領域に発見し，リバビリン誘導性貧血と関連する2つのSNPとの間に強い連鎖不平衡が認められた[49]（Table 5）．

4. 薬剤性肝障害

Dalyらはflucloxacillinを服用した333人の薬剤性肝障害に関するGWASによって，MHC領域のSNP（rs2395029）が有意に関連しており，

このSNPが*HLA-B*5701*と完全連鎖していることを報告した[50]．Singerらは1123人のCOX2選択的抑製薬lumiracoxib投与例による肝障害のGWASによって，MHC class II領域のSNPと肝障害の間に有意な関連性を認め，ハプロタイプ解析の結果，*HLA-DRB1*1501-HLA-DQB1*0602-HLA-DRB5*0101-HLA-DQA1*0102*が有意に関連するハプロタイプであったと報告している[51]．Lucenaらはヨーロッパ人733人によるamoxicillinの肝障害に関するGWASで，MHC領域のSNP（rs9274407）が薬剤性肝障害に関連し，このSNPは*HLA-DRB1*1501-DQB1*0602*のタグSNPであるrs3135388と連鎖していることを発見した[52]（Table 5）．

XI GWAS実施における注意点と問題点について

タイピングアレイの性能的個体差，アッセイ時の実験環境などによってタイピングの精度や成功率は変動し，実際の遺伝子型分布からの乖離も生じうる．異なるタイピングテクノロジーのプラットフォーム間では相違がより発生しやすい．より正確な結果を得るためには成功率やHardy-Weinberg平衡からのずれなどによって結果を選別（quality control check）することは重要である．さらにはケースとコントロールサンプルをランダムに配置するなどして，アッセイ間の誤差が関連解析に影響する可能性を低減するような配慮も推奨されている．また広い範囲の地域からサンプルを収集する場合には，集団構造化の影響を抑制するために各地域からケース・コントロール同じ割合でサンプリングしたり，地域別に層別解析して後で連結したほうがよい．コントロールサンプル（データ）の借用・使い回しも経済的な理由などから広く行われていることであるが，これも集団構造化やプラットフォーム間の特性差によって偽の関連性を生じる可能性もあるために注意を要する[53]．

よく吟味されたサンプルと研究デザインの関連解析によって同定報告された真に関連性を有する遺伝子多型であるにもかかわらずreplication studyが成功しにくい原因の1つとして，“winner's curse（勝者の呪い）”という原理が挙げられている[54]．この原理は，最初の関連解析研究報告における疾患感受性SNPのオッズ比などのエフェクトサイズの推定値は誇張される傾向があり，このことがしばしばreplication studyの研究デザインにおいて必要サンプルサイズを少なく見積もらせ，その結果としてreplication studyの結論に影響する可能性があるというものである．特に検出力の低い解析条件下の関連解析で真の効果を発見したときは，そのエフェクトサイズは誇張されやすい[54)55]．

多因子性疾患に対するGWASの理論的枠組みは，common disease common variant仮説[56]に基づいている．GWASは多因子性疾患の遺伝的要因の究明方法として当初非常に有望視され，多因子性疾患の遺伝素因の解明が進展するものと大いに期待された．しかし，多くの候補遺伝子が発見されて遺伝的要因の解明が進展したと思われる疾患・表現型においてさえ，発見された候補遺伝子全体をもってしても推測される遺伝的要因のごく一部のみしか説明されないということが報告されている．たとえばAllenらは，身長に関する3つのGWASの約18万人のメタアナリシスの結果，160個の関連遺伝子を同定したがそれら全体で説明しうるのは観察される遺伝性の20%のみであった[57]．またBarrettらは，Crohn病の3つのGWAS約8000人のメタアナリシスにより，既報の11の関連遺伝子の再現性確認と新規21関連遺伝子の発見をしたが，これら32遺伝子座全体で遺伝性の20%を説明できるのみであった[58]．これらの所見はcommon SNPを用いたGWAS解析の後もまだみつかっていない遺伝的要因がかなり残っていることを示唆しており，総称して“missing heritability（失われた遺伝性）”と呼ばれ，その本質についての議論がなされているところであるが，rare variant（アレル頻度の低いSNP）や構造多型（copy number variation），遺伝子間相互作用（epistasis），遺伝子環境間相互作用，DNAメチル化やヒストン修飾などの，epigeneticな調節機構などの関与が候補として挙げられている．

従来のGWASに続いて統計処理を加えることで検出力を向上させる試みがいくつか行われている．メタアナリシスは複数のエビデンスを統合するための確立された方法であるが，これを複数のGWASのデータに適用して統合することで検出力を高めることが可能で[59]，既に関連解析において広く普及してきている．さらには複数のSNPをセットにして解析するgene set analysisも研究されており，とりまとめることで検定の多重性を緩和し検出力の向上を目指すものである[60]．pathway analysisもその1つとみなすことができるが，こちらはある疾患の関連遺伝子は特定の経路上に集積している可能性が高いであろうという仮説に基づいている．また先述の遺伝子間相互作用をゲノムワイドに検索する方法も研究されている．

まとめ

common SNPを用いたアレイチップによる関連解析による網羅的な疾患関連遺伝子研究の時代になり，臨床上有用な新規遺伝子マーカーや創薬ターゲットが発見されている一方で，これまでのGWASによる成果では遺伝性の一部が説明できているに過ぎないこともわかってきた．今後遺伝要因検出の性能を向上しうる各種手法への多元的アプローチによって，未知の遺伝的リスクのさらなる解明につながっていくことが期待される．

本論文内容に関連する著者の利益相反
：なし

文献

1) Risch NJ : Searching for genetic determinants in the new millennium. Nature 405 ; 847–856 : 2000
2) Risch N, Merikangas K : The future of genetic studies of complex human diseases. Science 273 ; 1516–1517 : 1996
3) Yokosawa S, Yoshizawa K, Ota M, et al : A genomewide DNA microsatellite association study of Japanese patients with autoimmune hepatitis type 1. Hepatology 45 ; 384–390 : 2007
4) Hirschfield GM, Liu X, Xu C, et al : Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. N Engl J Med 360 ; 2544–2555 : 2009
5) Hirschfield GM, Liu X, Han Y, et al : Variants at IRF5-TNPO3, 17q12-21 and MMEL1 are associated with primary biliary cirrhosis. Nat Genet 42 ; 655–657 : 2010
6) Liu X, Invernizzi P, Lu Y, et al : Genome-wide meta-analyses identify three loci associated with primary biliary cirrhosis. Nat Genet 42 ; 658–660 : 2010
7) Mells GF, Floyd JA, Morley KI, et al : Genome-wide association study identifies 12 new susceptibility loci for primary biliary cirrhosis. Nat Genet 43 ; 329–332 : 2011
8) Nakamura M, Nishida N, Kawashima M, et al : Genome-wide association study identifies TNFSF15 and POU2AF1 as susceptibility loci for primary biliary cirrhosis in the Japanese population. Am J Hum Genet 91 ; 721–728 : 2012
9) Karlsen TH, Franke A, Melum E, et al : Genome-wide association analysis in primary sclerosing cholangitis. Gastroenterology 138 ; 1102–1111 : 2010
10) Melum E, Franke A, Schramm C, et al : Genome-wide association analysis in primary sclerosing cholangitis identifies two non-HLA susceptibility loci. Nat Genet 43 ; 17–19 : 2010
11) Folseraas T, Melum E, Rausch P, et al : Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. J Hepatol 57 ; 366–375 : 2012
12) Romeo S, Kozlitina J, Xing C, et al : Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 40 ; 1461–1465 : 2008
13) Yuan X, Waterworth D, Perry JR, et al : Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet 83 ; 520–528 : 2008
14) Chalasani N, Guo X, Loomba R, et al : Genome-wide association study identifies variants associated with histologic features of nonalcoholic Fatty liver disease. Gastroenterology 139 ; 1567–1576, 1576.e1–e6 : 2010
15) Speliotes EK, Yerges-Armstrong LM, Wu J, et al : Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet 7 ; e1001324 : 2011
16) Kawaguchi T, Sumida Y, Umemura A, et al : Genetic polymorphisms of the human PNPLA3 gene are strongly associated with severity of non-

alcoholic fatty liver disease in Japanese. PLoS One 7 ; e38322 : 2012
17) Adams LA, White SW, Marsh JA, et al : Association between liver-specific gene polymorphisms and their expression levels with nonalcoholic fatty liver disease. Hepatology 57 ; 590–600 : 2013
18) Kitamoto T, Kitamoto A, Yoneda M, et al : Genome-wide scan revealed that polymorphisms in the PNPLA3, SAMM50, and PARVB genes are associated with development and progression of nonalcoholic fatty liver disease in Japan. Hum Genet 132 ; 783–792 : 2013
19) Buch S, Schafmayer C, Völzke H, et al : A genome-wide association scan identifies the hepatic cholesterol transporter ABCG8 as a susceptibility factor for human gallstone disease. Nat Genet 39 ; 995–999 : 2007
20) Johnson AD, Kavousi M, Smith AV, et al : Genome-wide association meta-analysis for total serum bilirubin levels. Hum Mol Genet 18 ; 2700–2710 : 2009
21) Buch S, Schafmayer C, Völzke H, et al : Loci from a genome-wide analysis of bilirubin levels are associated with gallstone risk and composition. Gastroenterology 139 ; 1942–1951.e2 : 2010
22) Kamatani Y, Wattanapokayakit S, Ochi H, et al : A genome-wide association study identifies variants in the HLA-DP locus associated with chronic hepatitis B in Asians. Nat Genet 41 ; 591–595 : 2009
23) Mbarek H, Ochi H, Urabe Y, et al : A genome-wide association study of chronic hepatitis B identified novel risk locus in a Japanese population. Hum Mol Genet 20 ; 3884–3892 : 2011
24) Liu L, Li J, Yao J, et al : A genome-wide association study with DNA pooling identifies the variant rs11866328 in the GRIN2A gene that affects disease progression of chronic HBV infection. Viral Immunol 24 ; 397–402 : 2011
25) Png E, Thalamuthu A, Ong RT, et al : A genome-wide association study of hepatitis B vaccine response in an Indonesian population reveals multiple independent risk variants in the HLA region. Hum Mol Genet 20 ; 3893–3898 : 2011
26) Nishida N, Sawai H, Matsuura K, et al : Genome-wide association study confirming association of HLA-DP with protection against chronic hepatitis B and viral clearance in Japanese and Korean. PLoS One 7 ; e39175 : 2012
27) Patin E, Kutalik Z, Guergnon J, et al : Genome-wide association study identifies variants associated with progression of liver fibrosis from HCV infection. Gastroenterology 143 ; 1244–1252.e1–e12 : 2012
28) Urabe Y, Ochi H, Kato N, et al : A genome-wide association study of HCV-induced liver cirrhosis in the Japanese population identifies novel susceptibility loci at the MHC region. J Hepatol 58 ; 875–882 : 2013
29) Zhang H, Zhai Y, Hu Z, et al : Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. Nat Genet 42 ; 755–758 : 2010
30) Clifford RJ, Zhang J, Meerzaman DM, et al : Genetic variations at loci involved in the immune response are risk factors for hepatocellular carcinoma. Hepatology 52 ; 2034–2043 : 2010
31) Chan KY, Wong CM, Kwan JS, et al : Genome-wide association study of hepatocellular carcinoma in Southern Chinese patients with chronic hepatitis B virus infection. PLoS One 6 ; e28798 : 2011
32) Kumar V, Kato N, Urabe Y, et al : Genome-wide association study identifies a susceptibility locus for HCV-induced hepatocellular carcinoma. Nat Genet 43 ; 455–458 : 2011
33) Miki D, Ochi H, Hayes CN, et al : Variation in the DEPDC5 locus is associated with progression to hepatocellular carcinoma in chronic hepatitis C virus carriers. Nat Genet 43 ; 797–800 : 2011
34) Li S, Qian J, Yang Y, et al : GWAS identifies novel susceptibility loci on 6p21.32 and 21q21.3 for hepatocellular carcinoma in chronic hepatitis B virus carriers. PLoS Genet 8 ; e1002791 : 2012
35) Jiang DK, Sun J, Cao G, et al : Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. Nat Genet 45 ; 72–75 : 2013
36) Ge D, Fellay J, Thompson AJ, et al : Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461 ; 399–401 : 2009
37) Tanaka Y, Nishida N, Sugiyama M, et al : Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41 ; 1105–1109 : 2009
38) Suppiah V, Moldovan M, Ahlenstiel G, et al : IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41 ; 1100–1104 : 2009
39) Kawaoka T, Hayes CN, Ohishi W, et al : Predictive value of the IL28B polymorphism on the ef-

fect of interferon therapy in chronic hepatitis C patients with genotypes 2a and 2b. J Hepatol 54 ; 408–414 : 2011
40) Ochi H, Maekawa T, Abe H, et al : IL-28B predicts response to chronic hepatitis C therapy--fine-mapping and replication study in Asian populations. J Gen Virol 92 ; 1071–1081 : 2011
41) Hayes CN, Kobayashi M, Akuta N, et al : HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 60 ; 261–267 : 2011
42) Abe H, Hayes CN, Ochi H, et al : IL28 variation affects expression of interferon stimulated genes and peg-interferon and ribavirin therapy. J Hepatol 54 ; 1094–1101 : 2011
43) Ohnishi M, Tsuge M, Kohno T, et al : IL28B polymorphism is associated with fatty change in the liver of chronic hepatitis C patients. J Gastroenterol 47 ; 834–844 : 2012
44) Prokunina-Olsson L, Muchmore B, Tang W, et al : A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat Genet 45 ; 164–171 : 2013
45) Fellay J, Thompson AJ, Ge D, et al : ITPA gene variants protect against anaemia in patients treated for chronic hepatitis C. Nature 464 ; 405–408 : 2010
46) Ochi H, Maekawa T, Abe H, et al : ITPA polymorphism affects ribavirin-induced anemia and outcomes of therapy--a genome-wide study of Japanese HCV virus patients. Gastroenterology 139 ; 1190–1197 : 2010
47) Chayama K, Hayes CN, Abe H, et al : IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. J Infect Dis 204 ; 84–93 : 2011
48) Tanaka Y, Kurosaki M, Nishida N, et al : Genome-wide association study identified ITPA/DDRGK1 variants reflecting thrombocytopenia in pegylated interferon and ribavirin therapy for chronic hepatitis C. Hum Mol Genet 20 ; 3507–3516 : 2011
49) Thompson AJ, Clark PJ, Singh A, et al : Genome-wide association study of interferon-related cytopenia in chronic hepatitis C patients. J Hepatol 56 ; 313–319 : 2012
50) Daly AK, Donaldson PT, Bhatnagar P, et al : HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet 41 ; 816–819 : 2009
51) Singer JB, Lewitzky S, Leroy E, et al : A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury. Nat Genet 42 ; 711–714 : 2010
52) Lucena MI, Molokhia M, Shen Y, et al : Susceptibility to amoxicillin-clavulanate-induced liver injury is influenced by multiple HLA class I and II alleles. Gastroenterology 141 ; 338–347 : 2011
53) Lambert CG, Black LJ : Learning from our GWAS mistakes : from experimental design to scientific method. Biostatistics 13 ; 195–203 : 2012
54) Button KS, Ioannidis JP, Mokrysz C, et al : Power failure : why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci 14 ; 365–376 : 2013
55) Zollner S, Pritchard JK : Overcoming the Winner's curse : estimating penetrance parameters from case-control data. Am J Hum Genet 80 ; 605–615 : 2007
56) Lander ES : The new genomics : global views of biology. Science 274 ; 536–539 : 1996
57) Allen HL, Estrada K, Lettre G, et al : Hundreds of variants clustered in genomic loci and biological pathways affect human height. Nature 467 ; 832–838 : 2010
58) Barrett JC, Hansoul S, Nicolae DL, et al : Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 40 ; 955–962 : 2008
59) Yesupriya A, Yu W, Clyne M, et al : The continued need to synthesize the results of genetic associations across multiple studies. Genet Med 10 ; 633–635 : 2008
60) Fridley BL, Biernacka JM : Gene set analysis of SNP data : benefits, challenges, and future directions. Eur J Hum Genet 19 ; 837–843 : 2011

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LIVER

A Translational Study of Resistance Emergence Using Sequential Direct-Acting Antiviral Agents for Hepatitis C Using Ultra-Deep Sequencing

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OBJECTIVES: **Direct-acting antiviral agents (DAAs) against hepatitis C virus (HCV) have recently been developed and are ultimately hoped to replace interferon-based therapy. However, DAA monotherapy results in rapid emergence of resistant strains and DAAs must be used in combinations that present a high genetic barrier to resistance, although viral kinetics of multidrug-resistant strains remain poorly characterized. The aim of this study is to track the emergence and fitness of resistance using combinations of telaprevir and NS5A or NS5B inhibitors with genotype 1b clones.**

METHODS: **HCV-infected chimeric mice were treated with DAAs, and resistance was monitored using direct and ultra-deep sequencing.**

RESULTS: **Combination therapy with telaprevir and BMS-788329 (NS5A inhibitor) reduced serum HCV RNA to undetectable levels. The presence of an NS3-V36A telaprevir resistance mutation resulted in poor response to telaprevir monotherapy but showed significant HCV reduction when telaprevir was combined with BMS-788329. However, a BMS-788329-resistant strain emerged at low frequency. Infection with a BMS-788329-resistant NS5A-L31V mutation rapidly resulted in gain of an additional NS5A-Y93A mutation that conferred telaprevir resistance during combination therapy. Infection with dual NS5AL31V/NS5AY93H mutations resulted in poor response to combination therapy and development of telaprevir resistance. Although HCV RNA became undetectable soon after the beginning of combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor), rebound with emergence of resistance against all three drugs occurred. Triple resistance also occurred following infection with the NS3V36A/NS5AL31V/NS5AY93H triple mutation.**

CONCLUSIONS: **Resistant strains easily develop from cloned virus strains. Sequential use of DAAs should be avoided to prevent emergence of multidrug-resistant strains.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

According to the 2012 World Health Organization report, approximately 150 million individuals are chronically infected with hepatitis C virus (HCV) worldwide (1). Chronic HCV infection leads to chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma (1). Recently, two direct-acting antiviral agents (DAAs), telaprevir and boceprevir, have been approved for use in daily clinical practice to treat patients chronically infected with HCV genotype 1 (2–9). Triple therapy with peg-interferon, ribavirin, and either telaprevir or boceprevir has been reported to be the most effective approved treatment so far, with an eradication rate of 50 to 70%, compared with no >50% for combination therapy with peg-interferon and ribavirin alone (2–9). However, triple therapy is approved only for genotype 1, and many treated patients experience severe side effects that often result in early termination of the therapy (2–9). In an effort to establish safer

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and more effective therapies, a number of new DAAs are in development, and several have reached the clinical trial stage (10–12).

One of the two available drugs, telaprevir, has been approved in the United States, Canada, the Europe Union, and some Far East countries. Although telaprevir, a potent NS3/4A protease inhibitor, shows excellent antiviral activity, single use of the drug results in rapid emergence of resistant mutants (13–16). Twelve weeks of monotherapy with telaprevir resulted in a marked reduction of HCV RNA (16), and HCV RNA became undetectable in the serum in 3 of 10 treated patients (16). However, emergence of resistant mutants caused viral breakthrough and reappearance of the virus. To date, only one patient who showed a sustained virological response has been reported (16–18).

To avoid the emergence of resistance, combinations of DAAs targeting different viral protein with or without ribavirin have been investigated (19–22). We previously showed in animal experiments that HCV can be eradicated by only 4 weeks of combination therapy with telaprevir and NS5B inhibitor MK0608 (19). More recently, the combination of the NS3 protease inhibitor asunaprevir with the NS5A inhibitor daclatasvir was shown to eradicate the virus successfully in patients with genotype 1b (21,22). This interferon-free regimen has been reported to be more tolerable with few severe adverse events. Only some patients developed an elevation of transaminases and hyperbilirubinemia, probably due to the side effects of asunaprevir, which selectively accumulates in the liver with liver vs. plasma ratios ranging from 40-fold to 359-fold in several animal species (23). It has been reported that daclatasvir is effective even with very small doses and that the drug is excreted from the kidneys, suggesting that the liver damage seen in the combination therapy is not due to daclatasvir (22). As a significant portion of patients with HCV infection have already developed advanced liver diseases, the risk of coercing further liver damage should be minimized. Although it has been reported that telaprevir affects both liver and kidney transporters (24), no significant liver damage by telaprevir has been reported so far. This suggests that telaprevir is a good candidate for a future oral DAA combination therapy with daclatasvir.

We investigated in this study the effect of BMS-788329, a close analog of daclatasvir and telaprevir combination therapy against HCV genotypes 1b using a human hepatocyte chimeric mouse infection model. We also assessed how existing variants with resistant features affect response to DAA combination therapy. Furthermore, we investigated the possibility of multiple drug-resistant mutants using the combination of BMS-788329 and polymerase inhibitor BMS-821095. Our results showed that a mutant strain resistant against all three drugs emerges from clonal infection after sequential therapy with these drugs. Therefore, our results advocate for simultaneous DAA combination therapy, and we note the importance of resistance analysis and drug selection before therapy in order to successfully eradicate the virus.

METHODS

Animals

Generation of the uPA+/+/SCID+/+ mice and transplantation of human hepatocytes were performed as described previously (25). All mice were transplanted with frozen human hepatocytes obtained from the same donor. Animal protocols were approved by and performed in accordance with the guidelines of the local committee for animal experiments. Mice received humane care. Infection, extraction of serum samples, and killing were performed under ether anesthesia. Mice were inoculated intravenously with HCV-positive human serum samples and used for evaluation of drugs as reported previously (26,27). Mice were also prepared by injection of genotype 1b clone HCV-KT9 HCV RNA and its synthesized derivatives into the livers of chimeric mice (28).

Reagents

HCV-infected mice were perorally administered telaprevir based on 200 mg/kg body weight of (VX950; MP424; Mitsubishi Tanabe Pharma, Osaka, Japan), 10 mg/kg body weight of BMS-788329 (NS5A inhibitor), and 100 mg/kg body weight of BMS-821095 (NS5B inhibitor) (Bristol-Myers Squibb, New York, NY). These reagent doses were found to yield serum concentrations equivalent to those in treated human patients.

Human serum samples

Human serum samples containing a high titer of genotype 1b HCV ($2.2.\times10^6$–10^7 copies/ml) were obtained from patients with chronic hepatitis C after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Construction of mutant HCV strains and HCV RNA synthesis

We have previously reported infectious genotype 1b HCV clone HCV-KT9, which is able to replicate in human hepatocyte chimeric mice (27) (GenBank accession no. AB435162). HCV-KT9 mutants (V36A in NS3, L31V and Y93H in NS5A) were generated by site-directed mutagenesis using following primers: V36A in NS3, 5′-AGGTTCAAATAGCCTCCACCGCAACA-3′ (sense) and 5′-TGTTGCGGTGGAGGCTATTTGAACCT-3′ (antisense); L31V in NS5A, 5′-TCCAAACTCCTGCCGCGGGTACCGGGA GTCCCTTT-3′ (sense) and 5′-AAAGGGACTCCCGGTACCCG CGGCAGGAGTTTGGA-3′ (antisense); and Y93H in NS5A, 5′-AACATTCCCCATCAACGCACACACCACGGGCCCCTG CACA-3′ (sense) and 5′-TGTGCAGGGGCCCGTGGTGTGTGC GTTGATGGGGAATGTT-3′ (antisense). HCV RNA synthesis was performed as described previously (26,29). The RNA was analyzed using denaturing agarose gel electrophoresis and stored at −80 °C until use.

RNA extraction and HCV RNA quantification

RNA was extracted from mouse serum samples using Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in RNase-free water, and reverse transcribed using a random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (Rever-Tra Ace, Toyobo, Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer.

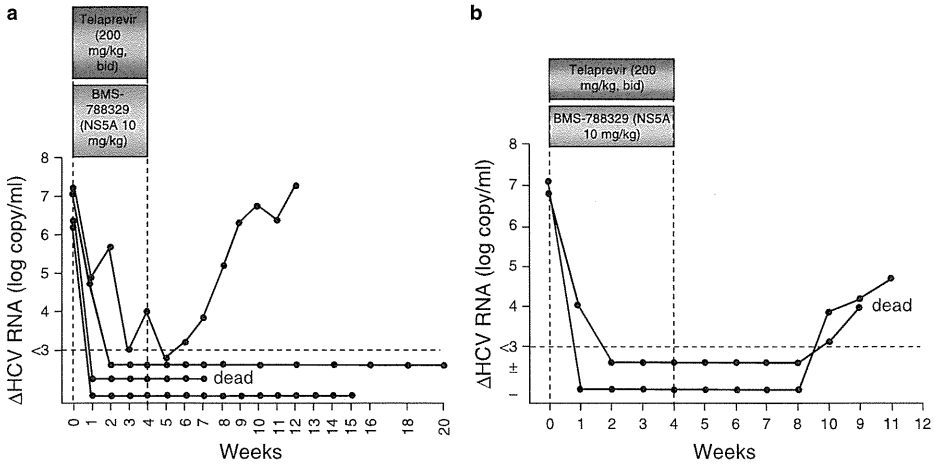

Figure 1. Effect of telaprevir plus NS5A inhibitor combination therapy for human hepatocyte chimeric mice infected with HCV genotype 1b. We established mice infected with serum from two different patients with genotype 1b and administrated 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). (**a**) Viral breakthrough occurred in one mouse infected with serum from the first patient. (**b**) Viral relapse occurred 5 weeks following the end of therapy in both mice infected with serum from the second patient. HCV, hepatitis C virus.

One microliter of complementary DNA were subjected to quantification of HCV RNA using 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA) (27).

Ultra-deep sequencing

We amplified 200–300-bp HCV complementary DNA fragments using KOD DNA Polymerase by nested PCR using the following primers: NS3 first set, 5′-CTGCATCATCACTAGCCTTACG-3′ (sense) and 5′-GAGCACCTTGTACCCTTGGGC-3′ (antisense); NS5A first set, 5′-ACTACGTGCCTGAGAGCGACG-3′ (sense) and 5′-CCAACCAGGTACTGATTGAGC-3′ (antisense); NS3 aa36, 5′-AGAACCAGGTCGAGGGAGAGG-3′ (sense) and 5′-AAGTAGAGGTCCGAGCTGCCG-3′ (antisense); NS3 aa155-156, 5′-GGGCACGTTGTGGGCATCTTC-3′ (sense) and 5′-GAGCACCTTGTACCCTTGGGC-3′ (antisense); NS5A aa31, 5′-TGGCTCCAGTCCAAACTCCTG-3′ (sense) and 5′-GGGAATGTTCCATGCCACGTG-3′ (antisense); NS5A aa93, 5′-TGGAACATTCCCCATCAACGC-3′ (sense) and 5′-CCAACCAGGTACTGATTGAGC-3′ (antisense) and then we performed end repair of fragmented DNA, adenine tailing of end repair, adaptor ligation, and PCR enrichment of adaptor-ligated DNA using TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA) according to the instructions provided by the manufacturer. Paired-end sequencing with multiplexed tags was carried out using Illumina Genome Analyzer IIx.

Direct sequencing

To compare the results of direct and ultra-deep sequencing, we performed direct sequencing using the same DNA fragments as ultra-deep sequencing. The primers for NS5B were as follows: NS5B first set, 5′-CGTCTGCTGCTCAATGTCCTAC-3′ (sense) and 5′-GTCATGCGGCTCACGGACCT-3′ (antisense); NS5B second set, 5′-GACTCAACGGTCACTGAGAG-3′ (sense) and 5′-CCTATTGGCCTGGAGTGTTT-3′ (antisense). Direct sequencing was carried out using a 3130 Genetic Analyzer (Life Technologies).

RESULTS

Effect of telaprevir plus NS5A inhibitor combination therapy for human hepatocyte chimeric mice infected with HCV genotype 1b

We inoculated six human hepatocyte chimeric mice with serum samples obtained from two patients with genotype 1b. After HCV RNA levels reached plateau, mice were administrated 200 mg/kg of telaprevir and 10 mg/kg of BMS-788329 (NS5A inhibitor) for 4 weeks (**Figure 1**). HCV RNA levels of three out of the four mice with serum from patient 1 decreased below the limit of detection (1.0×10^3 copies/ml). HCV RNA levels of the fourth mouse flared up before the end of therapy (viral breakthrough), and HCV RNA levels rapidly returned to pre-treatment levels following the end of therapy (**Figure 1a**). In the two mice inoculated with serum from patient 2, HCV levels remained negative for 4 weeks after drug withdrawal in both mice and then gradually increased to 1.0×10^5 copies/ml (**Figure 1b**). These results indicate that telaprevir plus NS5A inhibitor combination therapy at the above dose is effective against HCV genotype 1b (**Figure 1a,b**).

Combination treatment with telaprevir and BMS-788329 in human hepatocyte chimeric mice infected with an HCV clone containing NS3 V36A telaprevir resistance mutation

We established clonal infection with a telaprevir-resistant NS3 V36A mutant KT-9 strain in two human hepatocyte chimeric mice. Mice were treated with telaprevir alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus