its adjacently located ISDR work as adaptive mutations in the HCV replicon, possibly through decreasing the hyperphosphorylated form of NS5A,[37-40] which seems to control HCV replication. The conservation of c-terminal serine residual cluster of NS5A, downstream to IRRDR, is required for NS5A basal phosphorylation, interaction with the core protein on the lipid droplet, and thus virion formation.[41,42] Taken together, it can be speculated that the structural changes in core and NS5A protein can coordinately modify HCV replication, especially through virion formation around lipid droplets. However, the precise mechanism through which these modulations of viral proteins lead to the different treatment response should be further investigated.

In conclusion, we have found that polymorphic viral sequences in core aa.70, NS5A-ISDR aa.2224-2248, and NS5A-IRRDR aa.2340-2382 in genotype 1b HCV infection are correlated significantly with the treatment phase-specific viral responses to Peg-IFN/RBV therapy. In addition, these viral responses were also significantly correlated with the polymorphism in IL28B SNP, and this polymorphism was significantly correlated with the polymorphism in the core. More important, combined information of IL28B and IRRDR aa.2340-2382 is significantly important in predicting viral kinetics and treatment outcome. We consider that our comprehensive study provides a new basis for introducing Peg-IFN/RBV therapy as well as a new generation of anti-HCV therapies.

References

1. Lavanchy D. The global burden of hepatitis C. Liver Int 2009; 29(Suppl 1):74-81.
2. McHutchison JG, Lawitz EJ, Shiffman ML, Muir AJ, Galler GW, McCone J, et al. Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. N Engl J Med 2009;361:580-593.
3. Fried MW. The role of triple therapy in HCV genotype 1-experienced patients. Liver Int 2011;31(Suppl 1):58-61.
4. Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, Powell EE. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. Gut 2006;55:529-535.
5. Missiha S, Heathcote J, Arenovich T, Khan K; Canadian Pegasys Expanded Access G. Impact of asian race on response to combination therapy with peginterferon alfa-2a and ribavirin in chronic hepatitis C. Am J Gastroenterol 2007;102:2181-2188.
6. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
7. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon- and ribavirin therapy. Nat Genet 2009;41:1100-1104.
8. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon- and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
9. Zeuzem S, Rizzetto M, Ferenci P, Shiffman ML. Management of hepatitis C virus genotype 2 or 3 infection: treatment optimization on the basis of virological response. Antivir Ther 2009;14:143-154.
10. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995;96:224-230.
11. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, et al. HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 2011;60: 261-267.
12. Murphy MD, Rosen HR, Marousek GI, Chou S. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. Dig Dis Sci 2002;47:1195-1205.
13. Sarrazin C, Berg T, Lee JH, Ruster B, Kronenberger B, Roth WK, Zeuzem S. Mutations in the protein kinase-binding domain of the NS5A protein in patients infected with hepatitis C virus type 1a are associated with treatment response. J Infect Dis 2000;181:432-441.
14. Duverlie G, Khorsi H, Castelain S, Jaillon O, Izopet J, Lunel F, et al. Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. J Gen Virol 1998;79: 1373-1381.
15. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. HEPATOLOGY 2008;48:38-47.
16. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 1999;285:107-110.
17. Pfeiffer JK, Kirkegaard K. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. J Virol 2005;79:2346-2355.
18. Young KC, Lindsay KL, Lee KJ, Liu WC, He JW, Milstein SL, Lai MM. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. HEPATOLOGY 2003;38:869-878.
19. Hamano K, Sakamoto N, Enomoto N, Izumi N, Asahina Y, Kurosaki M, et al. Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy. J Gastroenterol Hepatol 2005;20:1401-1409.
20. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48: 372-380.
21. Donlin MJ, Cannon NA, Aurora R, Li J, Wahed AS, Di Bisceglie AM, et al. Contribution of genome-wide HCV genetic differences to outcome of interferon-based therapy in Caucasian American and African American patients. PLoS One 2010;5:e9032.
22. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211-8224.
23. Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol 2011;54:439-448.
24. Enomoto N, Maekawa S. HCV genetic elements determining the early response to peginterferon and ribavirin therapy. Intervirology 2010;53: 66-69.
25. Nagayama K, Kurosaki M, Enomoto N, Maekawa SY, Miyasaka Y, Tazawa J, et al. Time-related changes in full-length hepatitis C virus sequences and hepatitis activity. Virology 1999;263:244-253.

26. Lee SS, Ferenci P. Optimizing outcomes in patients with hepatitis C virus genotype 1 or 4. Antivir Ther 2008;13(Suppl 1):9-16.
27. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. HEPATOLOGY 2010;52: 421-429.
28. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, et al. HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 2010;60: 261-267.
29. Kurosaki M, Tanaka Y, Nishida N, Sakamoto N, Enomoto N, Honda M, et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. J Hepatol 2010;54:439-448.
30. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77-81.
31. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. HEPATOLOGY 2007;46: 1357-1364.
32. Miura M, Maekawa S, Kadokura M, Sueki R, Komase K, Shindo H, et al. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol Int 2011 Aug 17. doi no.: 10.1007/s12072–011-9307–6.
33. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089-1097.
34. Funaoka Y, Sakamoto N, Suda G, Itsui Y, Nakagawa M, Kakinuma S, et al. Analysis of interferon signaling by infectious hepatitis C virus clones with substitutions of core amino acids 70 and 91. J Virol 2011; 85:5986-5994.
35. Huang Y, Staschke K, De Francesco R, Tan SL. Phosphorylation of hepatitis C virus NS5A nonstructural protein: a new paradigm for phosphorylation-dependent viral RNA replication? Virology 2007;364: 1-9.
36. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 2004;85:2485-2502.
37. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol 2007;5:453-463.
38. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972-1974.
39. Maekawa S, Enomoto N, Sakamoto N, Kurosaki M, Ueda E, Kohashi T, et al. Introduction of NS5A mutations enables subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. J Viral Hepat 2004;11:394-403.
40. Kohashi T, Maekawa S, Sakamoto N, Kurosaki M, Watanabe H, Tanabe Y, et al. Site-specific mutation of the interferon sensitivity-determining region (ISDR) modulates hepatitis C virus replication. J Viral Hepat 2006;13:582-590.
41. Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, et al. Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J Virol 2008;82:7964-7976.
42. Tellinghuisen TL, Foss KL, Treadaway J. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog 2008;4:e1000032.

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

Donor hepatocytes†	No of chimeric mice	Inoculum	Test compound	Dose			
				Level (μg/kg)	Concentrtion (μg/ml)	Volume (ml/kg)	Frequency
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

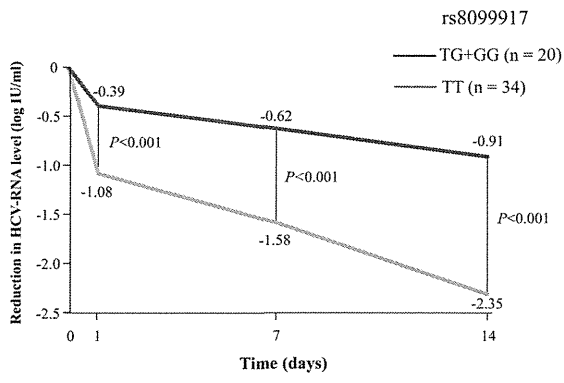

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

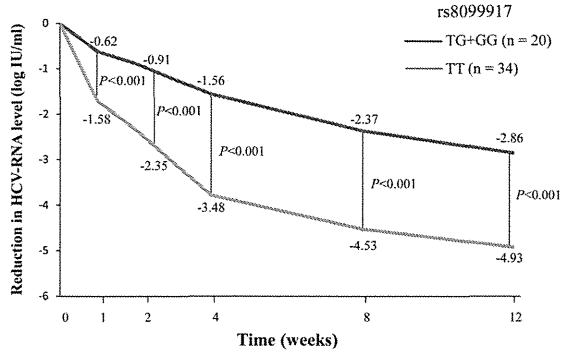

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

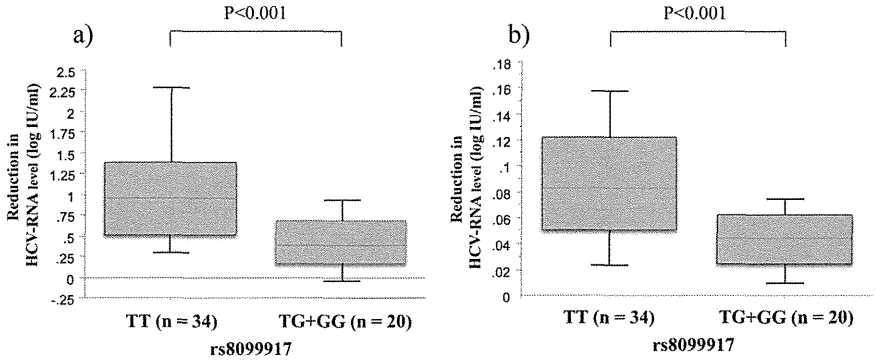

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

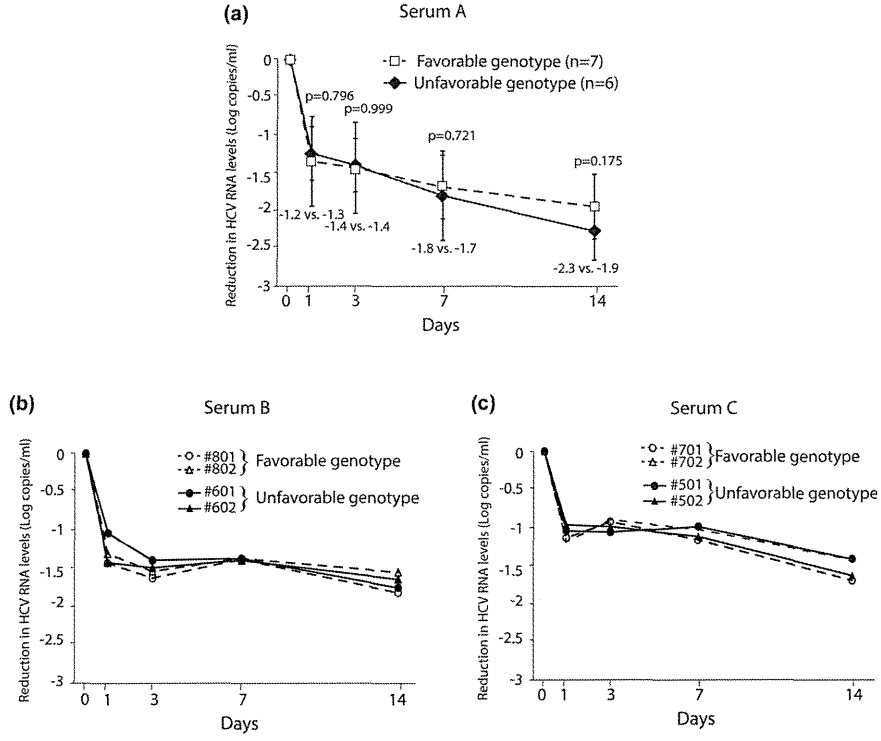

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.

(n=6) *IL28B* genotypes on days 1, 3, 7 and 14 (−1.2 vs −1.3, −1.4 vs −1.4, −1.8 vs −1.7, and −2.3 vs −1.9 log copies/ml) (figure 4A). Moreover, we prepared two additional serum samples from the other HCV-1b patients (serum B and C)[21] to confirm the influence of *IL28B* genotype in early viral kinetics during IFN treatment. After establishing persistent infection with new HCV-1b strains in all chimeric mice, they were also administered four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (figure 4B,C). In a similar fashion, no significant difference in HCV-RNA reduction in chimeric mice sera was observed between favourable and unfavourable *IL28B* genotypes.

Expression levels of ISG in chimeric mice livers

Because chimeric mice have the characteristic of severe combined immunodeficiency, the viral kinetics in chimeric mice sera during IFN treatment could be contributed by the innate immune response of HCV-infected human hepatocytes. Therefore, ISG expression levels in mice livers transplanted with human hepatocytes were compared between favourable and unfavourable *IL28B* genotypes (figure 5).

As shown in figure 5A, ISG expression levels in mice livers were measured at 8 h and 24 h after IFN treatment. The levels of representative antiviral ISG (eg, myxovirus resistance protein A, oligoadenylate synthetase 1, RNA-dependent protein kinase) and other ISG for promoting antiviral signalling (eg, Toll-like receptor 3, retinoic acid-inducible gene 1) were significantly induced at least 8 h after treatment, and prolonged at 24 h. No significant difference in ISG expression levels in HCV-infected livers was observed between favourable and unfavourable *IL28B* genotypes. The other inoculum for persistent infection of HCV-1b also demonstrated no significant difference in ISG

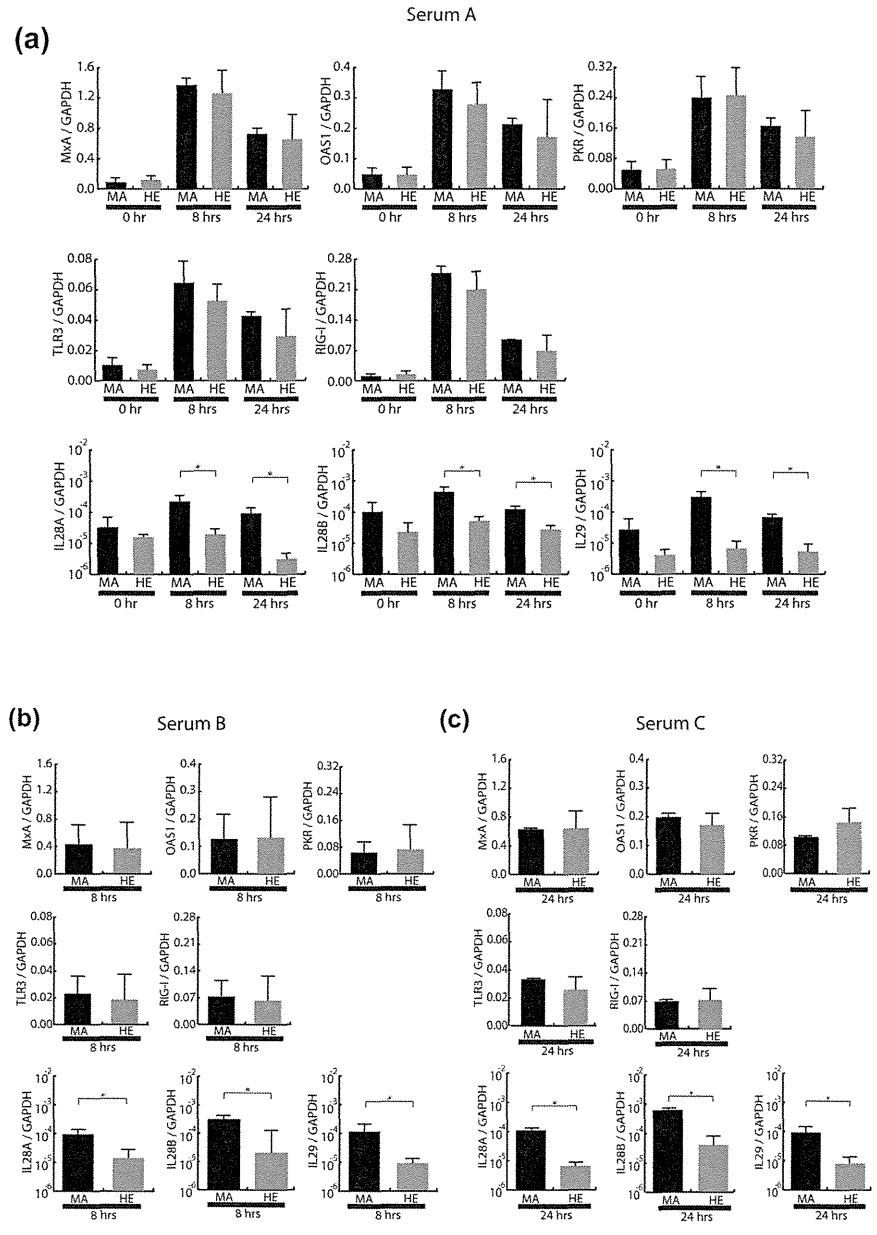

Figure 5 Intrahepatic interferon (IFN)-stimulated gene (ISG) expression levels in the pegylated interferon α (peg-IFN-α)-treated chimeric mice having human hepatocytes containing homozygous favourable allele (rs8099917 TT; MA) and heterozygous unfavourable allele (rs8099917 TG; HE) were measured and expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA. Data are represented as mean+SD. (A) Time kinetics of ISG after administration of the peg-IFN-α in serum A-infected chimeric mice (n=3, each genotype). Comparison of ISG expression levels at (B) 8 h in serum B-infected mice and (C) 24 h in serum C-infected mice after administering peg-IFN-α (n=3, each genotype). Predesigned real-time PCR assay of IL28B transcript purchased from Applied Biosystems can be cross-reactive to IL28A transcript. *p<0.05. MxA, myxovirus resistance protein A; OAS1, oligoadenylate synthetase 1; PKR, RNA-dependent protein kinase; RIG-1, retinoic acid-inducible gene 1; TLR3, Toll-like receptor 3.

expression levels between favourable and unfavourable *IL28B* genotypes (figure 5B,C). Interestingly, IFN-λ expression levels by treatment of peg-IFN-α were significantly induced in HCV-infected human hepatocytes harbouring the favourable *IL28B* genotype (figure 5 A–C).

DISCUSSION

Several recent studies have demonstrated a marked association between the chronic hepatitis C treatment response[6–9] and SNP (rs8099917, rs8103142 and rs12979860) near or within the region of the *IL28B* gene, which affected the viral dynamics during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals.[13]

It has been reported that when patients with chronic hepatitis C are treated by IFN-α or peg-IFN-α plus ribavirin, HCV-RNA generally declines after a 7–10 h delay.[25] The typical decline is biphasic and consists of a rapid first phase lasting for approximately 1–2 days during which HCV-RNA may fall 1–2 logs in patients infected with genotype 1, and subsequently a slower second phase of HCV-RNA decline.[26] The viral kinetics had a predictive value in evaluating antiviral efficacy.[14] In this study, biphasic decline of the HCV-RNA level during peg-IFN-α treatment was observed in both patients and chimeric mice infected with HCV genotype 1; however, in the first and second phases of viral kinetics, a difference between *IL28B* genotypes was observed only in HCV-infected patients; a more rapid decline in serum HCV-RNA levels after administering peg-IFN-α plus ribavirin was confirmed in patients with the TT genotype of rs8099917 compared to those with the TG/GG genotype.

On the other hand, in-vivo data using the chimeric mouse model showed no significant difference in the reduction of HCV-RNA titers in mouse serum among four different lots of human hepatocytes containing *IL28B* favourable (rs8099917 TT) or unfavourable (rs8099917 TG) genotypes, which was confirmed by the inoculation of two additional HCV strains. These results indicated that variants of the *IL28B* gene in donor hepatocytes had no influence on the response to peg-IFN-α under immunosuppressive conditions, suggesting that the immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

Two recent studies indeed revealed an association between the *IL28B* genotype and the expression level of hepatic ISG in human studies.[27 28] Quiescent hepatic ISG before treatment among patients with the *IL28B* favourable genotype have been associated with sensitivity to exogenous IFN treatment and viral eradication; however, it is difficult to establish whether the hepatic ISG expression level contributes to viral clearance independently or appears as a direct consequence of the *IL28B* genotype. Another recent study addressed this question and the results suggested that there is no absolute correlation with the *IL28B* genotype and hepatic expression of ISG.[29] Our results on the hepatic ISG expression level in immunodeficient chimeric mice also suggested that no significant difference in ISG expression levels was observed between favourable and unfavourable *IL28B* genotypes. However, these results were not consistent with a previous report using chimeric mice that the favourable *IL28B* genotype was associated with an early reduction in HCV-RNA by ISG induction.[30] The reasons for the discrepancy might depend on the dose and type of IFN treatment, as well as the time point when ISG expression was examined in the liver. In addition, although IFN-λ transcript levels measured in peripheral blood mononuclear cells or liver revealed inconsistent results in the context of an association with the *IL28B* genotype,[7 8] our preliminary assay on the IL28A, IL28B and IL29 transcripts in the liver first indicated that the induction of IFN-λ on peg-IFN-α administration could be associated with the *IL28B* genotype. Therefore, the induction of IFN-λ followed by immune response might contribute to different viral kinetics and treatment outcomes in HCV-infected patients, because no difference was found in chimeric mice without immune response.

It has also been reported that the mechanism of the association of genetic variations in the *IL28B* gene and spontaneous clearance of HCV may be related to the host innate immune response.[11] Interestingly, participants with seroconversion illness with jaundice were more frequently rs8099917 homozygous favourable allele (TT) than other genotypes (32% vs 5%, p=0.047). This suggests that a stronger immune response during the acute phase of HCV infection among patients with the *IL28B* favourable genotype would induce more frequent spontaneous clearance of HCV.

Taking into account both the above results in acute HCV infection and our results conducted on chimeric mice that have the characteristic of immunodeficiency, it is suggested that the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic hepatitis C patients would be composed of the intact immune system.

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Contributors YT and MM conceived the study. TW and FS and YT conducted the study equally. TW and FS coordinated the analysis and manuscript preparation. All the authors had input into the study design, patient recruitment and management or mouse management and critical revision of the manuscript for intellectual content. TW, FS and YT contributed equally.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of each ethics committee at the Nagoya City University and Nagasaki Medical Center (see supplementary information, available online only).

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REFERENCES

1. **Ray Kim W**. Global epidemiology and burden of hepatitis C. *Microbes Infect* 2002;**4**:1219–25.
2. **Foster GR.** Past, present, and future hepatitis C treatments. *Semin Liver Dis* 2004;**24**(Suppl. 2):97–104.
3. **Fried MW,** Shiffman ML, Reddy KR, *et al*. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;**347**:975–82.
4. **Manns MP,** McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;**358**:958–65.
5. **Mihm U,** Herrmann E, Sarrazin C, *et al*. Review article: predicting response in hepatitis C virus therapy. *Aliment Pharmacol Ther* 2006;**23**:1043–54.
6. **Ge D,** Fellay J, Thompson AJ, *et al*. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;**461**:399–401.
7. **Suppiah V,** Moldovan M, Ahlenstiel G, *et al*. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009;**41**:1100–4.
8. **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* 2009;**41**:1105–9.
9. **Rauch A,** Kutalik Z, Descombes P, *et al*. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* 2010;**138**:1338–45.

10. **Tanaka Y,** Nishida N, Sugiyama M, *et al*. lambda-Interferons and the single nucleotide polymorphisms: a milestone to tailor-made therapy for chronic hepatitis C. *Hepatol Res* 2010;**40**:449–60.
11. **Thomas DL,** Thio CL, Martin MP, *et al*. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009;**461**:798–801.
12. **Grebely J,** Petoumenos K, Hellard M, *et al*. Potential role for interleukin-28B genotype in treatment decision-making in recent hepatitis C virus infection. *Hepatology* 2010;**52**:1216–24.
13. **Thompson AJ,** Muir AJ, Sulkowski MS, *et al*. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 2010;**139**:120–29.
14. **Layden-Almer JE,** Layden TJ. Viral kinetics in hepatitis C virus: special patient populations. *Semin Liver Dis* 2003;**23**(Suppl. 1):29–33.
15. **Heckel JL,** Sandgren EP, Degen JL, *et al*. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 1990;**62**:447–56.
16. **Rhim JA,** Sandgren EP, Degen JL, *et al*. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994;**263**:1149–52.
17. **Tateno C,** Yoshizane Y, Saito N, *et al*. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;**165**:901–12.
18. **Mercer DF,** Schiller DE, Elliott JF, *et al*. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;**7**:927–33.
19. **Tsuge M,** Hiraga N, Takaishi H, *et al*. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005;**42**:1046–54.
20. **Kurbanov F,** Tanaka Y, Chub E, *et al*. Molecular epidemiology and interferon susceptibility of the natural recombinant hepatitis C virus strain RF1_2k/1b. *J Infect Dis* 2008;**198**:1448–56.
21. **Kurbanov F,** Tanaka Y, Matsuura K, *et al*. Positive selection of core 70Q variant genotype 1b hepatitis C virus strains induced by pegylated interferon and ribavirin. *J Infect Dis* 2010;**201**:1663–71.
22. **Inoue K,** Umehara T, Ruegg UT, *et al*. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 2007;**45**:921–8.
23. **Livak KJ,** Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402–8.
24. **Silver N,** Best S, Jiang J, *et al*. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol* 2006;**7**:33.
25. **Dahari H,** Layden-Almer JE, Perelson AS, *et al*. Hepatitis C viral kinetics in special populations. *Curr Hepat Rep* 2008;**7**:97–105.
26. **Neumann AU,** Lam NP, Dahari H, *et al*. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;**282**:103–7.
27. **Honda M,** Sakai A, Yamashita T, *et al*. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 2010;**139**:499–509.
28. **Urban TJ,** Thompson AJ, Bradrick SS, *et al*. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. *Hepatology* 2010;**52**:1888–96.
29. **Dill MT,** Duong FH, Vogt JE, *et al*. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology* 2011;**140**:1021–31.
30. **Hiraga N,** Abe H, Imamura M, *et al*. Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 2011;**54**:764–71.

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Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

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The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

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the world is divided conceptually into zones of high, intermediate, and low HBV endemic areas [Lavanchy, 2004]. In countries where the HBV infection is endemic, most infections result from the vertical transmission from the mother to the child in the peripartum period or from the infection in the early childhood. In the low HBV endemic regions, the neonatal or the childhood HBV infection is rare or even sporadic and the transmission of HBV occurs primarily among unvaccinated adults through the sexual transmission and injecting drug use [Custer et al., 2004].

Patients with chronic hepatitis B are considered to be the major reservoirs for the transmission of HBV. High incidence of infection with HBV is observed within the household contacts of chronic HBV carriers and it is not rare to have several members of the same household who have evidence of infection with HBV [Milas et al., 2000; Thakur et al., 2002]. However, the precise mechanisms of intra-familial spread have not been established clearly.

Different prophylactic strategies for controlling the HBV infection have been used by different countries depending on the prevalence of the HBV infection in each country [Poland and Jacobson, 2004]. The widespread immunization program against hepatitis B, which was implemented in more than 100 countries, was capable of dramatic reduction in the occurrence of chronic HBV infection and HCC [Zuckerman, 1997]. In Egypt, the HBV vaccine was included in 1992 in the Expanded Program of Immunization with injection at 2, 4, and 6 months of age [El Sherbini et al., 2006]. This program resulted in a significant reduction in the rate of acute symptomatic hepatitis B among the children in the age group eligible to receive the vaccine [Zakaria et al., 2007].

At least eight HBV genotypes have been identified based on the divergence of 8% or more of the entire nucleotide sequence and most of the HBV genotypes have a distinct geographical distribution [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Accumulated evidences indicated the difference in the virological characteristics among different HBV genotypes, which is reflected by the difference in the clinical outcome of infection with hepatitis B according to the infecting genotype [Miyakawa and Mizokami, 2003; Schaefer, 2005; Ozasa et al., 2006; Sugiyama et al., 2006]. However, data regarding the specificity of the transmission routes of each genotype is still scarce globally and need to be clarified.

The prevalence of HBV ranges between 2% and 6% in Egypt with the predominance of infection with HBV genotype D [Zekri et al., 2007]. It is widely known that Egypt is one of the countries with highest prevalence rate of infection with HCV in the world [el-Zayadi et al., 1992; Arthur et al., 1993; el Gohary et al., 1995]. However, the burden of HBV related progressive liver disease including liver cirrhosis and HCC in Egypt is observable either single or in a dual infection with HCV [Abdel-Wahab et al., 2000; el-Zayadi et al., 2005].

This study aimed to evaluate the prevalence of infection with HBV within the families of chronic HBV carriers in north Eastern Egypt. In addition, the intra-familial mode of transmission of HBV genotype D was also examined in the current cohort by the molecular evolutionary analyses. The impact of the HBV immunization programme in protecting this high-risk group was also investigated.

PATIENTS AND METHODS

Patients

The present study was conducted between January 2008 and June 2008 at the Communicable Disease Research and Training Centre, in Suez city. The study protocol was approved by the ethics committees of the participating institution and an informed consent was obtained from the included subjects.

Chronic HBV carriers were defined as individuals whose serum samples tested positive for HBsAg for at least 6-months period. Patients who fulfilled the criteria of chronic HBV carriers and were first detected within their families, were defined as the index cases ($n = 55$). The index cases included 40 (72.7%) men and 15 (27.3%) women. Their mean age ($\pm$ SD) was 41 ± 10.7 years and all the index cases were negative for HBeAg.

A total of 230 household contacts of the index cases were included in the study and defined as family members group. Data regarding their family relationship to the index cases, age, and the HBV vaccination history have been obtained.

According to the kinship of the family members to the index case group, the family members included 139 offspring, 4 parents, 46 spouses, 15 siblings, and 26 defined as other relatives who are living in the same house with the index cases.

Serological Methods

Serum samples were collected from the index cases and family members groups.

The Serum samples were examined for HBsAg, anti-HBc, anti-HBs, and HBeAg by the chemiluminescence enzyme immunoassay with the commercial assay kits (Fujirebio, Inc., Tokyo, Japan). The examination of the serum samples for anti-HCV and HIV was conducted using commercial kits (Abbott Laboratories, Abbott Park, IL).

Molecular Evolutionary Analysis

The HBV/DNA was extracted from 200 μl of serum samples positive for HBsAg using the QIAamp DNA MiniKit (QIGEN, Inc., Hilden, Germany), and re-suspended in 100 μl of a storage buffer (provided by the kit manufacturer).

The entire preS2 and S regions of the HBV genome (799 nucleotides; nucleotide positions 34–833) were amplified using the primers set and the conditions described previously [Sugauchi et al., 2001].

The amplified products were sequenced using Prism Big Dye (Pekrin–Elmer Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer according to the manufacturer's protocol. The sequences were aligned together with the CLUSAL X software programme [Thompson et al., 1994].

The phylogenetic tree was constructed using the neighbor joining method with Tamura-Nei's distance correction model using the Online Hepatitis Virus database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. The Bootstrap values were determined on 1000 database resampling tests. The sequences of other HBV isolates used for the construction of the phylogenetic tree were retrieved from the DDBJ/EMBL/GenBank sequence database and were indicated in their accession numbers. The new nucleotide sequences data that were reported in this manuscript will appear in the DDBJ/EMBL/GenBank sequence database with accession numbers AB561825-AB561856.

Statistical Analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent *t*-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL). *P*-values (two-tailed) <0.05 were considered to be significant statistically.

RESULTS

The family member included 96 (41.7%) males and 134 females (58.3%). Their mean age (±SD) was 20.6 ± 14.6. The rate of seropositivity for HBsAg and anti-HBc was 12.2% (28/230) and 23% (53/230) of the family members group with no statistical significant difference between the males and females members.

Age Group Distribution of HBV Infection Within the Family Members Group

Figure 1 illustrates the HBsAg and anti-HBc prevalences among different age groups of the family members. The highest prevalence of HBsAg seropositive cases was observed in the age group, 21–30 years old; (10/43; 23.3%) followed by the age group, 0–10 years old; (11/68; 16.2%). No statistical significant difference was found in the HBsAg seropositive rates between these two age groups. The prevalence of HBsAg was 7.7% (5/65), 3.4% (1/29), and 4% (1/25) in the age groups; 11–20, 31–40, and ≥41 years old, respectively. The prevalence of anti-HBc seropositive cases was significantly increasing with the age and the highest rate was observed in the age group ≥41 years old. The prevalence of anti-HBc was 8.8% (6/68), 20% (13/65), 25.6% (11/43), 37.9% (11/29), and 48% (12/25) in the age groups; 0–10, 11–20, 21–30, 31–40, and ≥41 years old, respectively.

The HBsAg and anti-HBc seropositive rates were analyzed in the family members with respect to their

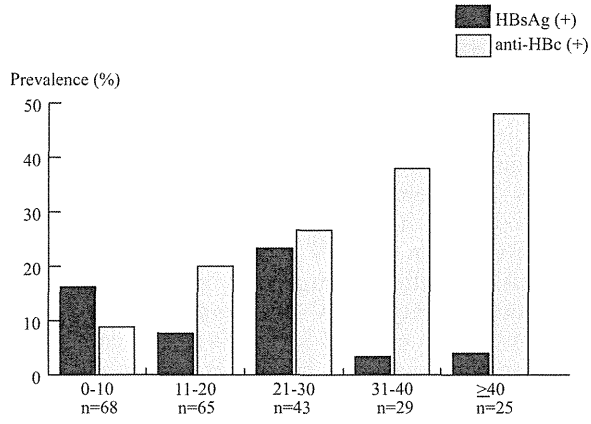

Fig. 1. Age distribution and HBV serological status among family members.

relationship to the index cases (Fig. 2A). As overall, the HBsAg was positive in 6.5% (3/46) spouse of index cases, 10.8% (15/139) of the offspring, 25% (1/4) of the parents, and 40% (6/15) of the siblings (Fig. 2A).

The prevalence of anti-HBc was 34.8% (16/46) in the spouse of index cases, 17.3% (24/139) in the offspring, 50% (2/4) in the parents, and 46.7% (7/15) in the siblings of the index cases (Fig. 2A).

Interestingly, the prevalence of HBsAg and anti-HBc was significantly higher in the family members of the females (19.2%, 15/78) than that of the males index cases (8.6%, 13/152; $P = 0.034$) and a trend of higher incidence of anti-HBc in the family members of the females than the males index cases (Fig. 2B). Among the offspring group, HBsAg and anti-HBc seropositive rates were significantly higher in the offspring of the females index cases (HBsAg; 23%, 11/47, anti-HBc; 29.8%, 14/47) cases than in the offspring of the males index cases (HBsAg; 4.3%, 4/92, antiHBc; 9.8%, 9/92), ($P = 0.001$, 0.003 respectively; Fig. 2C).

Further analysis was performed regarding the HBsAg seropositive rate in the offspring according to HBV infection of both one and two parents and the parent gender who is infected with HBV. Significantly higher rate of HBsAg positive (26.5%, 13/49) and anti-HBc positive (31.8%, 14/49) off spring were found in families where the mother was positive for HBsAg compared with families where the father was HBsAg positive (HBsAg; 4.7%, anti-HBc; 10.5%), ($P = 0.0006$, 0.009 respectively) (data not shown).

The seropositive rate of HBsAg was higher in the non-sexual contacts (13.6%, 25/184) of the index cases (parents, offspring, siblings, and cousins) than the sexual contacts (spouses; 6.5%, 3/46) with no statistical significant difference. Anti-HBc seropositive cases were observed more frequently in the sexual contacts (spouses) than in the non-sexual contacts (parents, offspring, siblings cousins) of the index cases. (Sexual vs. non-sexual contacts, 34.8% vs. 20.1%, $P = 0.049$) (data not shown).

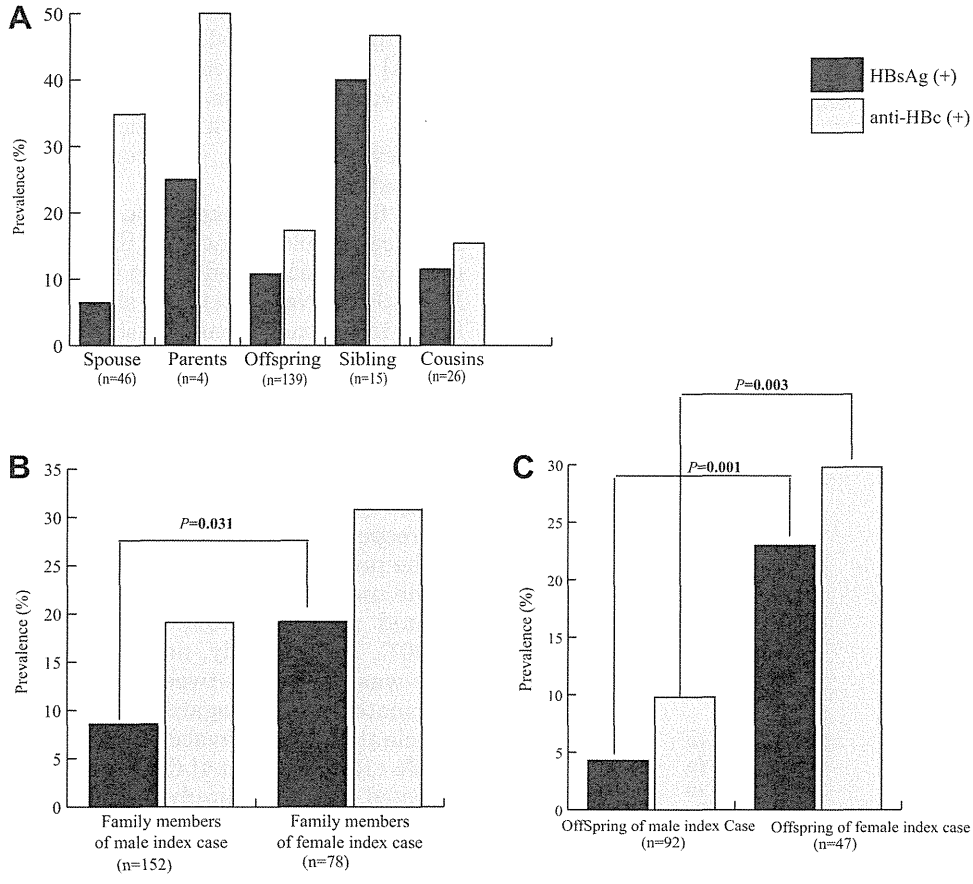

Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

Molecular Evolutionary Analysis and Transmission Pattern of Hepatitis B in the Family Members Group

Eighteen index cases out of 55 (32.7%) were found to have at least one family member positive for HBsAg. The age range of these index cases was 26–56 years and 50% (9/18) of them were male (Table I). Twenty-eight family members were found to be positive for HBsAg. The data regarding the degree of relativity of each family member infected with HBV to the index case, the age of the infected family member, and the vaccination status were indicated in Table I. The mean age (±SD) of the family members with active HBV infection was 17.8 ± 13.0 years old (Table I).

The HBV genomic region of 799-nt length and spanning PreS2 and S region was amplified in 44% (8/18) of the index cases and in 50% (14/28) of the family members infected with HBV. However, the target genomic region could be amplified and sequenced simultaneously in the index cases and their related family members in six subjects. These six subjects are defined in the present report, Table I and Figure 3 as F 3, F4, F19, F35, F37, and F 43 (Table I, Fig. 3).

To confirm the family clustering, a phylogentic tree was constructed by (1) the previous mentioned sequences (2) sequences isolated from the index cases whose family members were negative for HBsAg (3) HBV nucleotide sequences isolated from HBV chronic carriers residing in different districts in Egypt (North and South) either retrieved from the data base band or further included in the present study.

The phylogenetic analysis of the preS2 and S regions of the HBV genome revealed that the HBV isolates were of subgenotype D1 (Fig. 3). Using the phylogenetic analysis, in family 4 (F4), a high homology was detected between the HBV strains isolated from the grandmother together with her daughters and her grandchildren (Fig. 3). In the Family 35 and Family 43 (F35, and F43), the father and the child harbored very closely related HBV isolates and the phylogenetic analysis suggesting that the father may have been the source of infection for his child in Family 35 (F35) and Family 43 (F43). Similarly, very closely related HBV isolates were also detected in the

TABLE I. Descriptive Analysis of the Family Members Positive for the HbsAg

Subject	Relation (gender)	Age	HBV-vaccine[a]	PreS2 + S
F3	Index (F)	42		(+)
F3-1[b]	Daughter	13	Yes	(+)
F10	Index (F)	30		(−)
F10-1	Daughter	3	Yes	(+)
F11	Index (F)	33		(+)
F11-1	Daughter	8	Yes	(−)
F11-2	Cousin	10	Yes	(−)
F30	Index (F)	42		(−)
F30-1	Son	8	Yes	(−)
F34	Index (F)	30		(−)
F34-1	Son	7	Yes	(+)
F34-2	Son	9	Yes	(+)
F48	Index (F)	30		(−)
F48-1	Son	5	Yes	(−)
F35	Index (M)	29		(+)
F35-1[b]	Daughter	5	Yes	(+)
F39	Index (M)	33		(−)
F39-1	Daughter	5	Yes	(−)
F43	Index (M)	47		(+)
F43-1[b]	Daughter	12	Yes	(+)
F55	Index (M)	56		(+)
F55-1	Daughter	12	Yes	(−)
F37	Index (M)	45		(+)
F37-1[b]	Wife	26	Yes	(+)
F36	Index (M)	31		(−)
F36-1	Brother	26	No	(−)
F36-2	Brother	28	No	(−)
F36-3	Brother	22	No	(+)
F36-4	Mother	63	No	(+)
F4	Index (F)	54		(+)
F4-1	Daughter	35	No	(+)
F4-2	Daughter	20	No	(+)
F4-3	Grandchild	6	Yes	(+)
F4-4[b]	Grandchild	4	Yes	(+)
F19	Index (M)	29		(+)
F19-1[b]	Wife	27	No	(+)
F40	Index (M)	26		(−)
F40-1	Relative	24	No	(−)
F40-2	Relative	29	No	(−)
F41	Index (F)	53		(−)
F41-1	Daughter	23	No	(−)
F41-2	Daughter	17	No	(−)
F45	Index (M)	33		(+)
F45-1	Wife	27	No	(−)
F50	Index(F)	27		(−)
F50-1	Sister	25	No	(−)

[a]HBV vaccination history is provided for the family member.
[b]Index and family members who are positive simultaneously for the PreS2and S region.

man and his wife in Families 19 and 37 (F19 and F37) (Fig. 3). The molecular evolutionary analysis of the sequences isolated from the mother and her daughter in Family 3 (F3), yielded two separate but distinct groupings of the HBV isolates, suggesting that the presence of two different HBV viral isolates infecting the mother and her daughter (Fig. 3).

Serological Markers of HBV Infection in the Vaccinated and Unvaccinated Family Members

The family members group was subdivided into two subgroups according to the history of full regimen schedule of HBV vaccination as shown in Table II; (1) A group of vaccinated family members which includes a total of 142 subjects, who received the complete HBV vaccine regimen. (2) A group of unvaccinated family members, which included 88 subjects with no previous history or incomplete regimen of HBV vaccination.

The family members in the unvaccinated group were significantly older (mean ± SD; 32.5 ± 12.5 years old) than in the vaccinated group (mean ± SD; 13.3 ± 10.4, $P = 0.012$). No statistical significant difference was found in the male gender distribution between the two groups. The anti-HBs seropositive rate was significantly higher in the vaccinated group than the unvaccinated group [69.8% (99/142) vs. 33% (29/88), respectively, $P < 0.0001$] (Table II). The mean anti-HBs titre was significantly higher in the vaccinated than unvaccinated family members (70.1 ± 129.7 vs. 21.6 ± 51.7 mIU/ml, respectively $P < 0.0001$).

The prevalence of anti-HBc was significantly higher in the unvaccinated family members compared to vaccinated groups (37.5% vs. 14.1% respectively, $P < 0.0001$). Interestingly, no statistical significant difference was detected between the vaccinated and the unvaccinated groups regarding the prevalence of HBsAg [vaccinated vs. unvaccinated; 10.6% (15/142) vs. 14.8% (13/88), $P = 0.4$] (Table II). The HBV DNA was detected in 50% of family members positive for HBsAg with no statistical significant difference between the vaccinated (53%, 8/142) and unvaccinated groups (46.2%, 6/88) (Table II).

Mutations in the "a" determinant region. The available nucleotide sequences spanning the S gene of HBV isolated from the nine vaccinated and five unvaccinated members were translated into amino acid and aligned in correspondence to the reference sequences. The amino acid substitutions in the "a" determinant region that was reported to be associated with vaccine escape mutation were not detected. However, an amino acid substitution at the second loop of "a" determinant region (T143L) was clustered in the family subject F37 (F37 and F37-1) and found in one unvaccinated family member (F4-1). Another substitution was detected in the second loop of "a" determinant region (T140I) in an unvaccinated member (F36-1). P127A substitution in first loop of the "a" determinant region was clustered in the family 43(F43 and F43-1; Fig. 4).

DISCUSSION

The investigation of the intra-familial transmission in a particular region usually reveals valuable information about the routes of HBV spread in general and may help in exploring the HBV spread problem and local peculiarities. This study is the first one in Egypt done to explore the intra-familial spread of HBV infection and inclusively HBV genotype D transmission routes in Egypt. An evaluation of the impact of the universal HBV vaccination on the intra-familial transmission of HBV was also done.

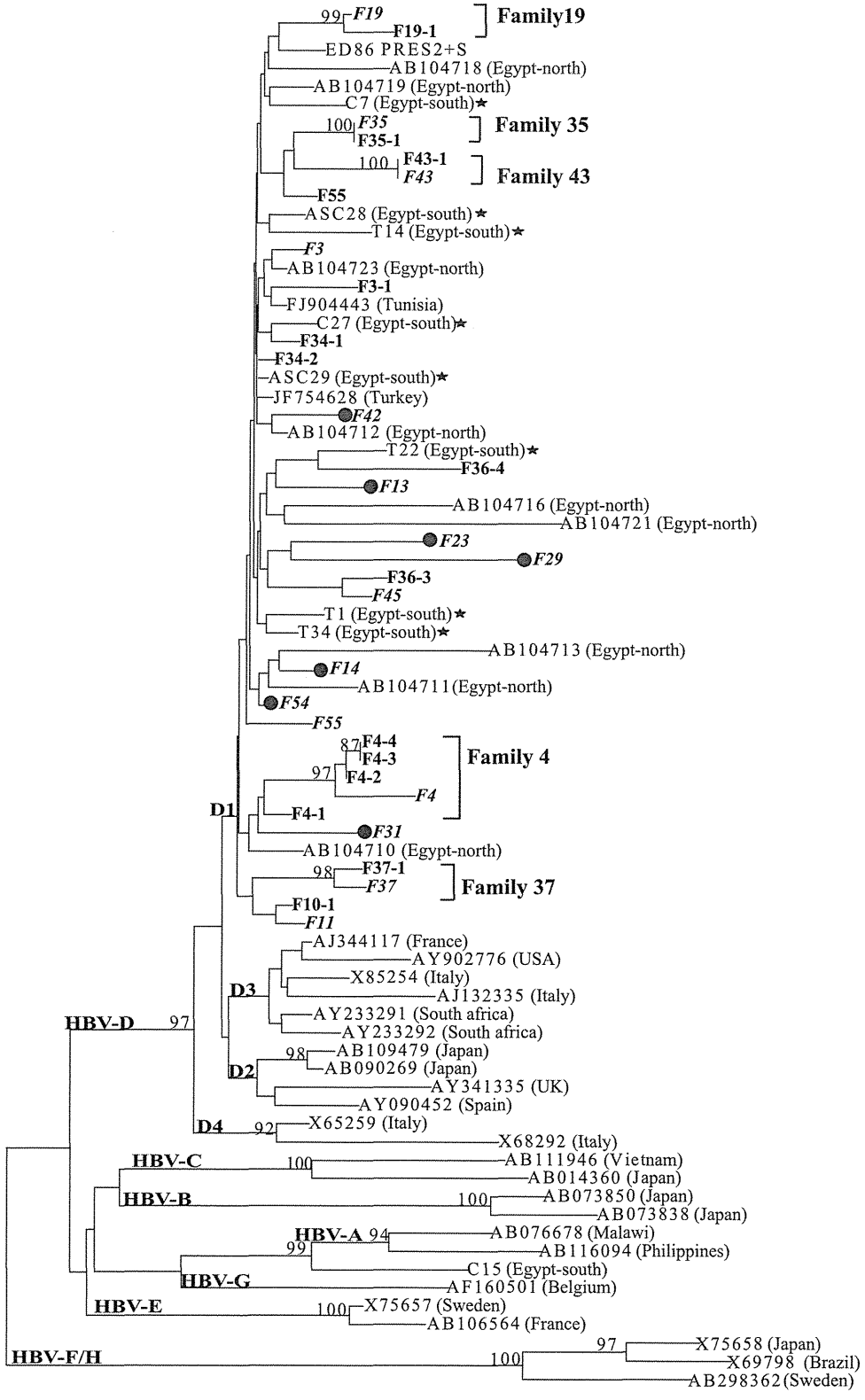

Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family members are indicated in italic bold and bold fonts respectively. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted in their accession numbers. Solid black rounds indicate sequences from index cases with family members negative for HBsAg. (★) Strains isolated from chronic hepatitis B carriers residing in Egypt south. The country origin of the reference sequences are indicated in brackets. HBV genotypes A–H are indicated in the cluster roots.

TABLE II. Comparison of Hepatitis B Serological Markers in Vaccinated Versus Unvaccinated Family Members Group

	Total (N = 230)	Vaccinated group (N = 142)	Unvaccinated group (N = 88)	*P*-value
Age[a]	20.6 ± 14.6	13.3 ± 10.4	32.5 ± 51.7	<0.0001
Gender (Male)[b]	96(41.7)	64 (45.1)	32 (36.4)	NS
Anti-HBc (+)[b]	53 (23)	20 (14.1)	33 (37.5)	<0.0001
HBsAg (+)[b]	28 (12.2)	15 (10.6)	13 (14.8)	NS
Anti-HBs (+)[b]	128 (55.7)	99 (69.8)	29(33)	<0.0001
HBV-DNA (+)[b]	14 (50)	8 (53.3)	6 (46.2)	NS

[a]Mean ± SD.
[b]N (%).

In the present study, 12.1% of the family members were infected with HBV. This incidence was much higher than that detected among the blood donors (1.4%) resident in the same area in Egypt (data not shown). Clustering of the HBV infection within the families has been described in nearby countries located within the same zone of the HBV endemicity but with different incidences; 30% in Turkey, 15.8% in Greece, and 11.9% in Iran [Alizadeh et al., 2005; Zervou et al., 2005; Ucmak et al., 2007]. An important risk factor was found to be implicated in acquiring the infection among the family was the presence of female infected with HBV. Furthermore, the higher incidence of HBsAg positive rate among the offspring of the females' index cases than that of males index cases illustrates clearly the role of the mother in the transmission of HBV. Similarly, Salkic et al. [2007] reported the same observation in his study from Bosnia [Salkic et al., 2007]. However, in Taiwan no significant difference was found in the HBsAg positivity among the offspring of the two groups, suggesting the importance of the paternal as well as the maternal transmission for the HBV intra-familial spread in Taiwan [Lin et al., 2005].

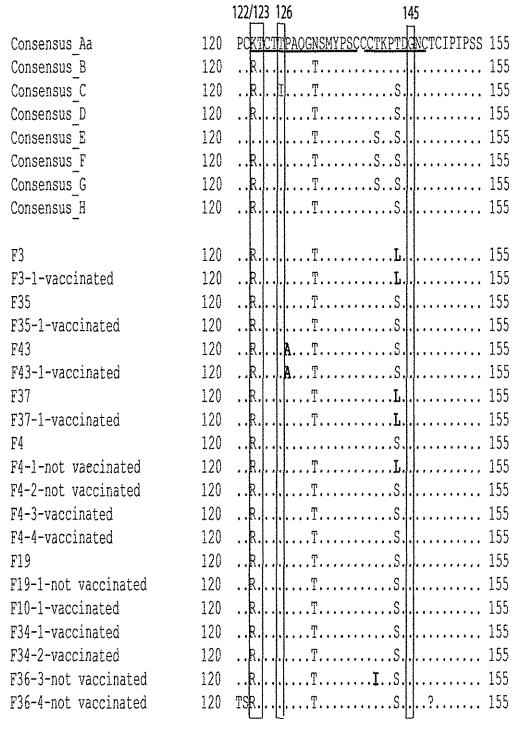

Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank database. Dots in alignment indicate identity of amino acids to the consensus sequence of genotype Aa/A1. First and second loop positions are underlined in the consensus sequence of the genotype Aa/A1 and positions of previously reported vaccine escape mutants are indicated in numbers and included in boxes.

Despite being a tedious and labor-intensive method, sequencing of the viral genomes isolated from different individuals, with the subsequent homology comparison and the phylogenetic analysis remains the golden approach for demonstrating the HBV transmission in a given population [Dumpis et al., 2001; Zampino et al., 2002; Tajiri et al., 2007].

The full length HBV sequence analysis is the gold standard for this purpose but remains a cost approach [Datta et al., 2007]. Highly variable HBV genomic region is recommended by some investigators to study the transmission event. Variability of the genomic region is affected by several factors one of which is the clinical characteristics of the studied cohort [Wu et al., 2005]. PreC/C region exhibit high variability in the cases of acute or fulminant hepatitis and thus analysis of this region is preferable for investigating the chain of recent/nosocomial fulminant cases [Bracho et al., 2006; Ozasa et al., 2006]. However, a high S gene variability is documented among the chronic hepatitis B carriers and their families, thus investigating the genotype, subgenotype, subtypes, and mutations by the sequence analysis of the S gene with further analysis by testing the constructed phylogenetic tree with the bootstrap resampling maximum-likelihood test, may provide enough confidence to prove the transmission event in the case of chronic HBV carriers [Thakur et al., 2003]. Hence, in the present study, the phylogenetic analysis of the HBV nucleotide sequences spanning the entire preS2 and S HBV genomic regions and isolated from chronic hepatitis B carriers which include index cases and their family members revealed the infection with HBV genotype D which coincides with the previous

data regarding the predominance of infection with HBV genotype D in Egypt [Saudy et al., 2003]. In addition, the phylogenetic analysis documented the presence of three different patterns of HBV genotype D transmission within the families in Egypt; maternal transmission (from mother to child as in the family 4), paternal transmission (from father to child as in family 35 and family 43) and spousal transmission (between spouses as in family 19 and family 37). This was different from the transmission pattern characteristics of genotype D in Uzbekistan where the horizontal transmission was the predominant route of infection with HBV genotype D within a family [Avazova et al., 2008].

The Data regarding the difference of transmission routes of HBV infection between different genotypes are controversial and scarce. Based on the findings that the patients infected with HBV genotype C may exhibit delayed HBeAg seroconversion decades later than the patients infected with other genotypes, Livingston et al. [2007] speculated that genotype C is the most responsible for the perinatal transmission and that the other genotypes (A, B, D, and F) are mainly transmitted horizontally [Livingston et al., 2007]. A recent study has shown a different data through exploring that both genotypes B and C can be transmitted by maternal and horizontal routes [Wen et al., 2011]. Whether different HBV genotypes have different transmission routes remains a question, which needs further global studies to clarify this interesting and important issue.

In an attempt to evaluate the influence of the universal vaccination on the intra-familial HBV infection, it was surprising to find a high prevalence rate of HBsAg among the vaccinated members with no significant difference when compared to the unvaccinated group. In an agreement with the present data, El Sherbini et al. [2006] reported the unchangeable prevalence of HBsAg among the vaccinated school children across a decade despite the significant decrease of the anti-HBc rate [El Sherbini et al., 2006]. The possible explanation for this vaccine failure is the acquiring of the HBV infection in the lag period between the birth and the time of receiving the first HBV vaccine dose at the age of 2 months. Supporting our explanation is the recent data coming from Taiwan where a different HBV infection prophylactic strategy is applied by administrating the first dose of the HBV vaccine at birth with the administration of the hepatitis B immunoglobulin to the infants born to the HBeAg positive mother within 24 hr after birth. The recent study has clearly demonstrated that the current HBV prophylactic strategy in Taiwan was capable of reducing the intra-familial HBV transmission and reducing the overall HBsAg positive rate among the infants [Mu et al., 2011]. In Japan, the extension of the active and passive immunization to the babies born to HBeAg negative mother had greatly reduced the HBsAg prevalence to 0.2% of blood donors younger than 19 years old [Noto et al., 2003; Matsuura et al., 2009]. The present study recommends the changing of the current HBV prophylactic policy in Egypt. It would be needed to provide the first dose of the HBV vaccine at birth together with screening for HBV infection markers prenatally and administration of the HBIG to the infants born from HBeAg-positive mothers. The documented role of the HBV spousal transmission in the present study by the phylogenetic analysis (Family 19 and Family 37), coincides with the recent data conducted in Egypt that the first sexual contact with an infected spouse was a significant risk factor for infection with HBV among females and may further emphasize the importance of the premarital screening for HBV in Egypt [Paez Jimenez et al., 2009]. Investigating the " a" determinant region of viral isolates retrieved from the vaccinated members infected with HBV provides no evidence of breakthrough infection by previously reported vaccine escape mutant virus [Carman et al., 1990].

In conclusion, the present study has clearly explored the role of the HBV intra-familial transmission and spread in north Eastern Egypt. Three patterns of HBV transmission were determined in the current cohort infected with HBV genotype D; maternal, paternal, and spousal. The present study recommends the change of the current prophylactic policy against the HBV infection in Egypt by including the first dose of HBV vaccine at birth, screening of pregnant women for HBsAg and the administration of HBIG to the infants born from HBeAg positive mothers within 24 hr after birth. Further studies are needed globally to determine the transmission patterns of different HBV genotypes and locally in different districts in Egypt to explore the impact of familial transmission in HBV infection in Egypt.

REFERENCES

Abdel-Wahab M, el-Enein AA, Abou-Zeid M, el-Fiky A, Abdallah T, Fawzy M, Fouad A, Sultan A, Fathy O, el-Ebidy G, elghawalby N, Ezzat F. 2000. Hepatocellular carcinoma in Mansoura-Egypt: Experience of 385 patients at a single center. Hepatogastroenterology 47:663–668.

Alizadeh AH, Ranjbar M, Ansari S, Alavian SM, Shalmani HM, Hekmat L, Zali MR. 2005. Intra-familial prevalence of hepatitis B virologic markers in HBsAg positive family members in Nahavand, Iran World J Gastroenterol 11:4857–4860.

Arthur RR, el-Sharkawy MS, Cope SE, Botros BA, Oun S, Morrill JC, Shope RE, Hibbs RG, Darwish MA, Imam IZ. 1993. Recurrence of Rift Valley fever in Egypt. Lancet 342:1149–1150.

Avazova D, Kurbanov F, Tanaka Y, Sugiyama M, Radchenko I, Ruziev D, Musabaev E, Mizokami M. 2008. Hepatitis B virus transmission pattern and vaccination efficiency in Uzbekistan. J Med Virol 80:217–224.

Bracho MA, Gosalbes MJ, Gonzalez F, Moya A, Gonzalez-Candelas F. 2006. Molecular epidemiology and evolution in an outbreak of fulminant hepatitis B virus. J Clin Microbiol 44:1288–1294.

Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC. 1990. Vaccine-induced escape mutant of hepatitis B virus. Lancet 336:325–329.

Chen DS. 1993. From hepatitis to hepatoma: Lessons from type B viral hepatitis. Science 262:369–370.

Custer B, Sullivan SD, Hazlet TK, Iloeje U, Veenstra DL, Kowdley KV. 2004. Global epidemiology of hepatitis B virus. J Clin Gastroenterol 38:S158–S168.

Datta S, Banerjee A, Chandra PK, Chakravarty R. 2007. Selecting a genetic region for molecular analysis of hepatitis B virus transmission. J Clin Microbiol 45:687; author reply 688.

Dumpis U, Holmes EC, Mendy M, Hill A, Thursz M, Hall A, Whittle H, Karayiannis P. 2001. Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. J Hepatol 35:99–104.

el Gohary A, Hassan A, Nooman Z, Lavanchy D, Mayerat X, el Ayat A, Fawaz N, Gobran F, Ahmed M, Kawano F, Ragheb M, Elkady A, Tanaka Y, Murakami S, Attia FM, Hassan AA, Hassan MF, Shedid MM, Abdel Reheem HB, Khan A, Mizokami M. 1995. High prevalence of hepatitis C virus among urban and rural population groups in Egypt. Acta Trop 59:155–161.

El Sherbini A, Mohsen SA, Seleem Z, Ghany AA, Moneib A, Abaza AH. 2006. Hepatitis B virus among schoolchildren in an endemic area in Egypt over a decade: Impact of hepatitis B vaccine. Am J Infect Control 34:600–602.

el-Zayadi A, Selim O, Rafik M, el-Haddad S. 1992. Prevalence of hepatitis C virus among non-A, non-B-related chronic liver disease in Egypt. J Hepatol 14:416–417.

el-Zayadi AR, Badran HM, Barakat EM, Attia Mel D, Shawky S, Mohamed MK, Selim O, Saeid A. 2005. Hepatocellular carcinoma in Egypt: A single center study over a decade. World J Gastroenterol 11:5193–5198.

Kao JH, Chen DS. 2002. Global control of hepatitis B virus infection. Lancet Infect Dis 2:395–403.

Lavanchy D. 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 11:97–107.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Lin CL, Kao JH, Chen BF, Chen PJ, Lai MY, Chen DS. 2005. Application of hepatitis B virus genotyping and phylogenetic analysis in intrafamilial transmission of hepatitis B virus. Clin Infect Dis 41:1576–1581.

Livingston SE, Simonetti JP, Bulkow LR, Homan CE, Snowball MM, Cagle HH, Negus SE, McMahon BJ. 2007. Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. Gastroenterology 133:1452–1457.

Matsuura K, Tanaka Y, Hige S, Yamada G, Murawaki Y, Komatsu M, Kuramitsu T, Kawata S, Tanaka E, Izumi N, Okuse C, Kakumu S, Okanoue T, Hino K, Hiasa Y, Sata M, Maeshiro T, Sugauchi F, Nojiri S, Joh T, Miyakawa Y, Mizokami M. 2009. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. J Clin Microbiol 47:1476–1483.

Milas J, Ropac D, Mulic R, Milas V, Valek I, Zoric I, Kozul K. 2000. Hepatitis B in the family. Eur J Epidemiol 16:203–208.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

Mu SC, Wang GM, Jow GM, Chen BF. 2011. Impact of universal vaccination on intrafamilial transmission of hepatitis B virus. J Med Virol 83:783–790.

Norder H, Courouce AM, Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. J Gastroenterol Hepatol 18:943–949.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, Kuramitsu T, Suzuki K, Tanaka E, Okada S, Tokita H, Asahina Y, Inoue K, Kakumu S, Okanoue T, Murawaki Y, Hino K, Onji M, Yatsuhashi H, Sakugawa H, Miyakawa Y, Ueda R, Mizokami M. 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 44:326–334.

Paez Jimenez A, El-Din NS, El-Hoseiny M, El-Daly M, Abdel-Hamid M, El Aidi S, Sultan Y, El-Sayed N, Mohamed MK, Fontanet A. 2009. Community transmission of hepatitis B virus in Egypt: Results from a case–control study in Greater Cairo. Int J Epidemiol 38:757–765.

Poland GA, Jacobson RM. 2004. Clinical practice: Prevention of hepatitis B with the hepatitis B vaccine. N Engl J Med 351:2832–2838.

Salkic NN, Zildzic M, Muminhodzic K, Pavlovic-Calic N, Zerem E, Ahmetagic S, Mott-Divkovic S, Alibegovic E. 2007. Intrafamilial transmission of hepatitis B in Tuzla region of Bosnia and Herzegovina. Eur J Gastroenterol Hepatol 19:113–118.

Saudy N, Sugauchi F, Tanaka Y, Suzuki S, Aal AA, Zaid MA, Agha S, Mizokami M. 2003. Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. J Med Virol 70: 529–536.

Schaefer S. 2005. Hepatitis B virus: Significance of genotypes. J Viral Hepat 12:111–124.

Seeger C, Mason WS. 2000. Hepatitis B virus biology. Microbiol Mol Biol Rev 64:51–68.

Shin IT, Tanaka Y, Tateno Y, Mizokami M. 2008. Development and public release of a comprehensive hepatitis virus database. Hepatol Res 38:234–243.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 81: 67–74.

Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: Complete genome sequence and phylogenetic relatedness. J Gen Virol 82:883–892.

Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, Gish RG, Kramvis A, Shimada T, Izumi N, Kaito M, Miyakawa Y, Mizokami M. 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44:915–924.

Tajiri H, Tanaka Y, Kagimoto S, Murakami J, Tokuhara D, Mizokami M. 2007. Molecular evidence of father-to-child transmission of hepatitis B virus. J Med Virol 79:922–926.

Thakur V, Guptan RC, Malhotra V, Basir SF, Sarin SK. 2002. Prevalence of hepatitis B infection within family contacts of chronic liver disease patients – Does HBeAg positivity really matter? J Assoc Physicians India 50:1386–1394.

Thakur V, Kazim SN, Guptan RC, Malhotra V, Sarin SK. 2003. Molecular epidemiology and transmission of hepatitis B virus in close family contacts of HBV-related chronic liver disease patients. J Med Virol 70:520–528.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Ucmak H, Faruk Kokoglu O, Celik M, Ergun UG. 2007. Intra-familial spread of hepatitis B virus infection in eastern Turkey. Epidemiol Infect 135:1338–1343.

Wen WH, Chen HL, Ni YH, Hsu HY, Kao JH, Hu FC, Chang MH. 2011. Secular trend of the viral genotype distribution in children with chronic hepatitis B virus infection after universal infant immunization. Hepatology 53:429–436.

Wu W, Chen Y, Ruan B, Li LJ. 2005. Gene heterogeneity of hepatitis B virus isolates from patients with severe hepatitis B. Hepatobiliary Pancreat Dis Int 4:530–534.

Zakaria S, Fouad R, Shaker O, Zaki S, Hashem A, El-Kamary SS, Esmat G, Zakaria S. 2007. Changing patterns of acute viral hepatitis at a major urban referral center in Egypt. Clin Infect Dis 44:e30–e36.

Zampino R, Lobello S, Chiaramonte M, Venturi-Pasini C, Dumpis U, Thursz M, Karayiannis P. 2002. Intra-familial transmission of hepatitis B virus in Italy: Phylogenetic sequence analysis and amino-acid variation of the core gene. J Hepatol 36:248–253.

Zekri AR, Hafez MM, Mohamed NI, Hassan ZK, El-Sayed MH, Khaled MM, Mansour T. 2007. Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virol J 4:74.

Zervou EK, Gatselis NK, Xanthi E, Ziciadis K, Georgiadou SP, Dalekos GN. 2005. Intrafamilial spread of hepatitis B virus infection in Greece. Eur J Gastroenterol Hepatol 17:911–915.

Zuckerman AJ. 1997. Prevention of primary liver cancer by immunization. N Engl J Med 336:1906–1907.

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A computational procedure for identifying master regulator candidates: a case study on diabetes progression in Goto-Kakizaki rats

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Abstract

Background: We have recently identified a number of active regulatory networks involved in diabetes progression in Goto-Kakizaki (GK) rats by network screening. The networks were quite consistent with the previous knowledge of the regulatory relationships between transcription factors (TFs) and their regulated genes. To study the underlying molecular mechanisms directly related to phenotype changes, such as diseases, we also previously developed a computational procedure for identifying transcriptional master regulators (MRs) in conjunction with network screening and network inference, by effectively perturbing the phenotype states.

Results: In this work, we further improved our previous method for identifying MR candidates, by listing them in a more reliable manner, and applied the method to reveal the MR candidates for diabetes progression in GK rats from the active networks. Specifically, the active TF-gene pairs for different time periods in GK rats were first extracted from the networks by network screening. Another set of active TF-gene pairs was selected by network inference, by considering the gene expression signatures for those periods between GK and Wistar-Kyoto (WKY) rats. The TF-gene pairs extracted by the two methods were then further selected, from the viewpoints of the emergence specificity of TF in GK rats and the regulated-gene coverage of TF in the expression signature. Finally, we narrowed all of the genes down to only 5 TFs (Etv4, Fus, Nr2f1, Sp2, and Tcfap2b) as the candidates of MRs, with 54 regulated genes, by merging the selected TF-gene pairs.

Conclusions: The present method has successfully identified biologically plausible MR candidates, including the TFs related to diabetes in previous reports. Although the experimental verifications of the candidates and the present procedure are beyond the scope of this study, we narrowed down the candidates to 5 TFs, which can be used to perform the verification experiments relatively easily. The numerical results showed that our computational method is an efficient way to detect the key molecules responsible for biological phenomena.

Background

Recent developments in genome-wide computational analyses have successfully identified causal interactions [1], and showed promise in the identification of dysregulated genes in development and tumor progression pathways [2]. For example, a computational analysis procedure was applied to identify the MRs causally linked to the activation of a specific gene set, mesenchymal gene expression signature (MGES), in human malignant glioma [3]. Indeed, 53 TFs were obtained by the

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ARACNe algorithm and the MGES enrichment test, and among them, the top 6 TFs with the largest fraction of MGES genes were experimentally controlled, as the MR candidates. Finally, 2 of the top 6 TFs, STAT3 and CEBPB, were experimentally verified as MRs of mesenchymal transformation. Unfortunately, the computational method employed in the previous work was unsophisticated and required further improvement. For example, it is unclear why the method selected the top 6 TFs from 53 TFs, rather than 5 or 7 TFs. Although the coverage of the TFs for the MGES genes was carefully considered, there was no rational criterion for the final selection of the MR candidates. Furthermore, ARACNe considers the relationships between three genes for selecting MR candidates. However, there are some well known mathematical techniques that consider multiple relationships and have been applied to infer regulatory networks [4].

We previously reported 39 candidates of active networks for diabetes progression in the Goto-Kakizaki rat (GK), which were identified by network screening, in comparison with the Wistar-Kyoto (WKY) rat [5]. Network screening is a procedure to extract the regulatory networks activated under particular conditions, based on the known regulatory networks [5-7]. The candidates were characterized by the known biological pathways that were consistent with the previous knowledge about diabetes. Unfortunately, the plausibility of the active networks could not be verified experimentally. This was partly because the results were presented in a metaphysical form, and as the biological pathway, instead of the list of concrete target genes. Actually, the active networks were composed of many genes that were not amenable to experimental verification.

To overcome these problems, we recently developed a procedure for identifying MR candidates, by a combination of network screening and network inference [8]. The network screening strongly depends on the previous knowledge of the regulatory networks. To compensate for the limitations of network screening, we introduced a network inference method, which is a version of a path consistency algorithm (PC-A) [9] or a modified PC-A [10,11] that applies PC-A to biological data with high redundancy. The performance of our procedure was tested for MRs in human malignant glioma, using the same data set in ref. [3], and worked well [8]. In total, 22 TFs and 27 TFs were detected by the network screening and the network inference, respectively, and 3 TFs overlapped between them. Interestingly, 2 of the 3 TFs were STAT3 and CEBPB, which were verified experimentally as the master regulators in the previous report [3].

In this paper, we sought to identify the candidates of master regulators for diabetes progression, using the spontaneous diabetic GK rat model. Based on the networks specific to diabetes progression identified in our previous report [5] and the networks inferred by the modified PC-A, we intended to narrow down the candidate molecules responsible for diabetes further, by identifying the master regulators that play a central role in diabetes progression in GK rats. Furthermore, we improved our previous method [8] to narrow down the candidates in a more reliable manner, by considering the coverage of a TF for its regulated genes in a statistical manner, in addition to the specificity of the TF to the target biological phenomena. As expected from the previous case of the computational identification of MRs in a human brain tumor [8] and the present improvements, we identified a limited set of reliable MR candidates, and thus provided information for further experimental design for candidate verification.

Results

Overview of our computational procedure

In our computational procedure, we identified MR candidates by two approaches, which are schematically shown in Figure 1. One is a knowledge-based approach, which estimates the consistency of the network structures among the known networks with the measured data (named "network screening") [5-7]. The other is a data-driven inference approach, which estimates the conditional independency between the genes by calculating the partial correlation coefficients (named "modified path consistency algorithm") [10,11]. In both cases, we further selected the MR candidates by considering the enrichment of the gene expression signature in the networks. Finally, we refined the candidates by considering the TF specificity and the regulated-gene coverage. The details are described in the Methods.

MR candidates detected by network screening

In our previous study [5], we used network screening to identify 39 networks for GK and WKY rats in three growth periods: 4w, from 8w to 12w, and from 16w to 20w, among the 1,760 networks in the reference network set. Based on these results, we further selected the MR candidates. From the 39 networks, in total, we extracted 568 binary relationships of TFs and their regulated genes, which were specifically found in the three periods for GK and WKY rats, under the condition that the gene expression shows a difference with a false discovery rate (FDR) of less than 0.05, between the two strains of rats for each period (see the details in the Methods). The numbers of genes specifically found in each period in GK and WKY rats are as follows: 54 genes at the period of 4w in GK; 199 at 8w and 12w in GK; 56 at 16w and 20w in GK; 95 at the period of 4w in WKY; 125 at 8w and 12w in WKY; and 39 at 16w and 20w in WKY. Note that some TF-