Antiviral Research 94 (2012) 54–56

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short communication

Predicting sustained viral response to hepatitis C using a rapid and simple IL28B rs8099917 genotyping assay

Wei Li [a,1], Yanli Zeng [a,1], Junjie Wang [a,1], Bin Zhou [a], Jian Zhang [a], Hao Zhang [a], Jingtao Li [a], Yingsong Wu [b], Rifat Hamoudi [c,*], Yuanping Zhou [a,*]

[a] Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou, China
[b] School of Bio-Technology, Southern Medical University, Guangzhou, China
[c] Department of Pathology, Rockefeller Building, University College London, London, WC1E 6JJ, United Kingdom

ARTICLE INFO

Article history:
Received 26 December 2011
Revised 3 February 2012
Accepted 13 February 2012
Available online 22 February 2012

Keywords:
Hepatitis C Virus
IL28B
Treatment response
Single nucleotide polymorphism

ABSTRACT

Recent studies showed that two single nucleotide polymorphisms (SNPs) (rs12979860 and rs8099917) near the gene IL28B coding for IFNλ3 were associated with the antiviral treatment response of the combination therapy of pegIFN plus RBV. We established the use of tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR) for detecting IL28B rs8099917 genotype (T > G) in 56 Chinese chronic hepatitis C patients infected with Hepatitis C Virus (HCV) genotype 1. The new assay showed 98.2% specificity, and was confirmed by direct sequencing. Among the 56 samples, TT genotype and TG genotype accounted for 80.4% (45/56) and 19.6% (11/56), respectively. GG genotype was not found. The proportion of responders in TT group was higher than that in TG group (68.9% vs. 27.3%, $p = 0.029$). For HCV clinical decision-making, using the new assay, rs8099917 genotyping could provide similar information to rs12979860 genotyping due to a strong association between the two SNPs in Chinese patients. The assay system in this study can be implemented using basic laboratory equipments, making it convenient for clinical and research purposes.

Hepatitis C Virus (HCV) infection is a major cause of chronic liver disease, with an estimated 170 million individuals chronically infected around the world. The combination of pegylated interferon (pegIFN) and ribavirin (RBV) is the current standard of care (SOC) therapy for chronic hepatitis C (CHC) (Ghany et al., 2009), but only approximately 50% of patients can achieve sustained virological response (SVR) (Hoofnagle and Seeff, 2006). In light of the side effects and high costs of SOC, it is important to identify factors involved in predicting an individual's response before treatment.

Recently, genome-wide association studies (GWASs) showed that single nucleotide polymorphism (SNP) (rs12979860 or rs8099917) near the gene IL28B coding for IFNλ3 was associated with the antiviral treatment response of the combination therapy of pegIFN plus RBV (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Rauch et al., 2010). The predictive value of IL28B polymorphism testing for SVR to pegIFN /RBV in HCV genotype 1 patients is superior to that of the pre-treatment HCV at the RNA level, fibrosis stage, age, and sex (Thompson et al., 2010).

For IL28B rs8099917 genotyping, although real-time polymerase chain reaction (RT-PCR) might be more reliable (Ito et al., 2011), it needs special equipments and has other idiosyncrasies such as specific TaqMan probes. Thus, PCR-restriction fragment length polymorphism (PCR-RFLP) for detecting IL28B rs8099917 genotype appeared to be suitable for epidemiological investigations (Nakamoto et al., 2011), however it might misdiagnose genotypes in the case of enzyme incomplete digestions of products. Here, we developed a simple and effective tetra-primer amplification refractory mutation system PCR (ARMS-PCR) for detecting IL28B rs8099917 genotype (T > G), which could be implemented using standard laboratory equipments.

The ARMS-PCR has been reported as a rapid, reliable, simple and economical assay for SNP genotyping (Ye et al., 2001; Okayama et al., 2004; S, 2001). Some studies considered that the method was more effective by the base mismatch of the 3′-terminal nucleotide in combination with a second deliberate mismatch near 3′-terminus base in both the inner primers (Ye et al., 2001; S, 2001). However, the ARMS-PCR using the double-base mismatch

Abbreviations: HCV, Hepatitis C Virus; RBV, ribavirin; SOC, standard of care; CHC, chronic hepatitis C; SVR, sustained virological response; GWAS, genome-wide association study; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; ARMS, tetra-primer amplification refractory mutation system; EVR, early virological response.

* Corresponding authors. Address: Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China. Tel.: +86 20 61641945; fax: +86 20 87719653 (Y. Zhou).

E-mail addresses: r.hamoudi@ucl.ac.uk (R. Hamoudi), yuanpingzhou@163.com (Y. Zhou).

[1] These authors contributed equally.

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2012.02.007

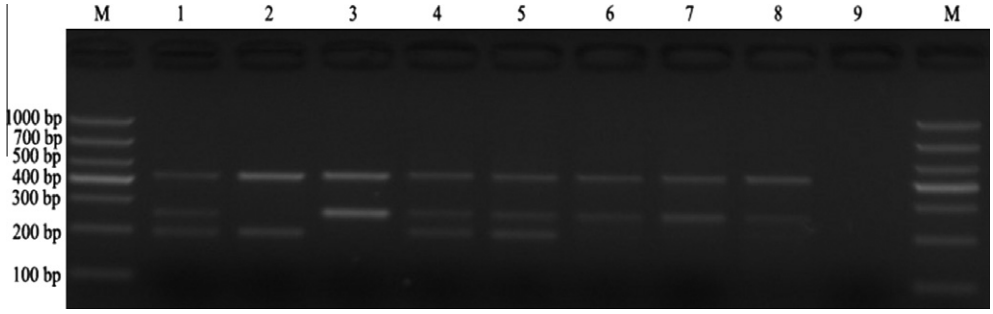

Fig. 1. The electrophoresis pattern of IL28B genotyping by the ARMS-PCR. DNA marker (M) shows approximate products size of different genotypes: TG: 419 bp, 258 bp and 199 bp, TT: 419 bp and 258 bp, GG: 419 bp and 199 bp. Lane 1–3 (positive control): TG, GG and TT; Lane 4, 5 (samples): TG; Lane 6–8 (samples): TT; Lane 9: negative control.

Table 1
Clinical characteristics of 56 patients infected with HCV genotype 1 according to the status of rs8099917 genotype.

	Total(n = 56)	rs8099917 genotype		P
		TT(n = 45)	TG(n = 11)	
Age (mean ± SD)	37.7 ± 10.9	38.6 ± 11.7	36.9 ± 9.1	0.338
Male/female	29/27	25/20	4/7	0.253
Responser (%)	34(60.7)	31(68.9)	3(27.3)	0.029
Non-responser (%)	22(39.3)	14(31.1)	8(72.7)	

Notes: Student's t test and Chi-square test were used to test the univariate data, and $p < 0.05$ was considered statistically significant. Responders were defined as patients who completed a 48 week course of PEG-IFN-a/RBV combination therapy and achieved sustained virological response (SVR). Non-responders were defined as those who did not achieve SVR or did not receive further treatment due to them not achieving early virological response (EVR).

primers might show the lower amplification efficiency of inner primers, which leads to lower sensitivity and weak product brand of the allele-specific amplification. The primers in this study were designed according to published IL28B rs8099917 genomic sequence. The two outer primers amplified both wild-type and mutant alleles, and the two inner primers amplified specifically either the wild-type or the mutant allele. To obtain the optimal inner primers, we designed them using the single- or double-base mismatch at the 3′-terminal nucleotide. To decrease the non-specific allele amplification of the mismatch primer, we used the following strategies: Firstly, adjusting the ratio of the inner primers concentration relative to that of the outer primers (the ratio of outer/inner primers: 1/2); Secondly, elevating the annealing temperature. We found that the ARMS-PCR using the single-base mismatch primers was effective in determining the IL28B rs8099917 genotype, comparing to that using double-base mismatch primers. The primer sequences were as follow. Outer: 5′-CATCCCTCATCC CACTTCT-3′ (forward) and 5′-AAAGGCTTCTGGTATCAAC-3′ (reverse). Inner: (5′-TTCCTTTCTGTGAGCAATG-3′) (G allele) and (5′-TGGTTCCAATTTGGGTGAA-3′) (T allele).

The PCR was carried out in a single tube containing 30 μL of a reaction buffer made up of the following components: 50 ng of genomic DNA, 0.15 μM of each of the outer primers and 0.3 μM of each of the inner primers, a 0.2 mM concentration of each of the four deoxynucleotides, 1.5U of Hotstart DNA polymerase (Takara, Dalian, China), and 10× PCR buffer containing 1.5 mM MgCl2. The PCR was performed by first denaturing the samples for 5 min at 94 °C, followed by 35 cycles consisting of 94 °C for 30 s, 58 °C for 25 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. PCR products were electrophoresed on a 3% agarose gels.

To evaluate the specificity of the ARMS-PCR, three IL28B rs8099917 genotype plasmids (TT, TG and GG) clones were used as positive control to ensure that it could identify simultaneously

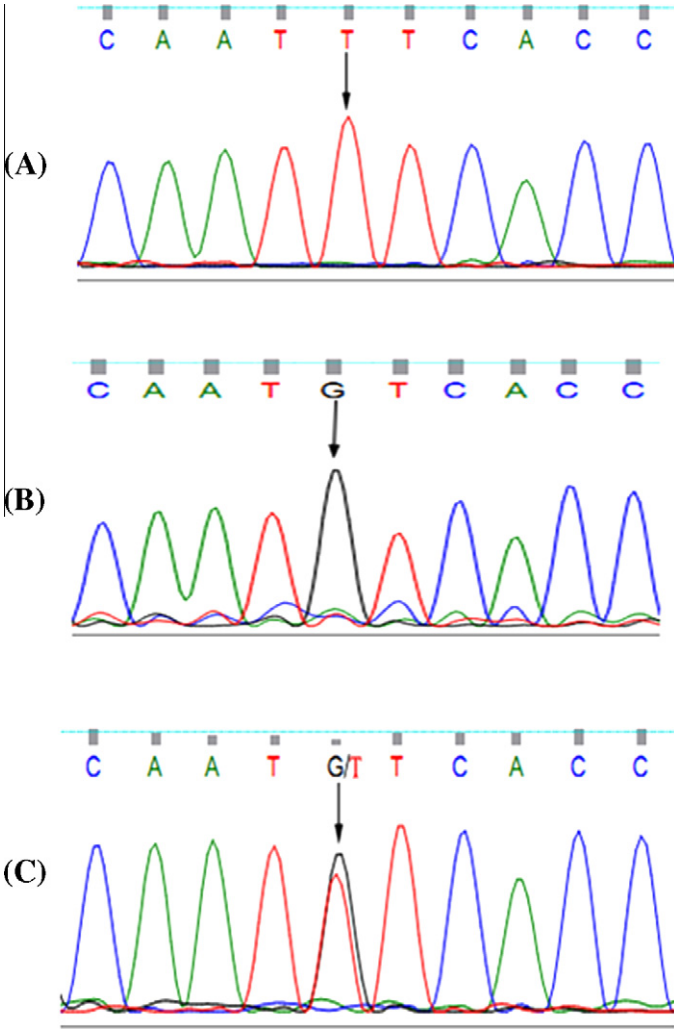

Fig. 2. The chromatograms of direct sequencing for IL28B rs8099917 genotype. (A) Sequence chromatogram of TT genotype. (B) Sequence chromatogram of GG genotype. (C) Sequence chromatogram of TG genotype: double peaks at SNP position.

these genotypes. The electrophoresis pattern of the positive control plasmids genotyping was shown in Fig. 1. A sample identified as genotype TG by sequencing was employed to construct plasmids of different genotypes. Firstly, the outer primers were used to amplify the template of the genotype TG sample. The amplified prod-

uct of 419 bp in size comprised two kinds of fragments: one for allele T and another for G. Secondly, the mixed allele products were cloned using *pMD18-T* Simple Vector (Takara, Dalian, China), and then chemically transformed into *Escherichia coli JM109* (Takara, Dalian, China). Thus, the TT and GG plasmids were constructed using positive clones. Finally, the TG plasmids were made by mixing the TT and GG plasmids in equal ratio.

The ARMS-PCR was effective and specific in determining the IL28B rs8099917 genotypes. The electrophoresis pattern of the genotyping samples was also shown in Fig. 1. IL28B rs8099917 genotypes were successfully determined in all patients using the new assay. Among 56 patients with chronic HCV-1 infection, TT genotype and TG genotype accounted for 80.4% (45/56) and 19.6% (11/56), respectively. GG genotype was not found. The proportion of responder patients in TT group was higher than that in TG group (68.9% vs. 27.3%, $p = 0.029$) (Table 1), indicating that minor allele G was the most important factor in predicting poor response to the combination therapy of pegIFN /RBV. The genotyping results of the ARMS-PCR were confirmed by direct Sanger sequencing (Invitrogen, Shanghai, China) of amplified PCR products using the outer primers in all samples, and showed correspondence with that by DNA sequencing in 55 samples (98.2%). Fig. 2 showed the sequencing chromatograms of rs8099917 genotypes.

For IL28B rs12979860 genotyping, unfortunately, we could not find the optimal primer pair conditions to effectively differentiate variants due to high GC content of the sequence near the SNP making it difficult to design optimal inner primer pairs. Strong linkage disequilibrium between rs12979860 and rs8099917 was reported in Asian population (Sinn et al., 2011), thus we developed the new assay for rs8099917 genotyping. We used direct sequencing to detect rs12979860 genotypes in the same cohort, and also found the strong linkage disequilibrium between the two SNPs in Chinese population (data not shown). Although an ARMS-PCR based method for rs12979860 genotyping was reported earlier (Galmozzi et al., 2011), we found that rs8099917 genotyping could provide similar information to rs12979860 genotyping due to the strong association between the two SNPs in Chinese patients.

The association of IL28B rs8099917 variations with IFN-based treatment response has been shown in different ethnic cohorts by GWAS study (Tanaka et al., 2009; Rauch et al., 2010; Suppiah et al., 2009), suggesting that TT genotype is the good response variation while TG/GG genotype is the poor response variation. The present study demonstrated that TT genotype accounted for the major proportion, and the responders in TT group was higher than that in TG group among Chinese patients with HCV-1 infection. The study showed the frequency of favorable allele (SNP rs12979860 and rs8099917) was higher in Asian than that in Caucasian population (Balagopal et al., 2010). So the higher favorable allele frequency might explain the reason that Asians have a higher likelihood of achieving SVR than Caucasians (Yu and Chuang, 2009).

In conclusion, we developed a rapid, simple and reliable assay, allowing the identification of IL28B rs8099917 genotyping to predict the response to IFN-based treatment. This assay system could be a convenient tool for epidemiological and clinical investigations.

Acknowledgments

This study was supported by grant from the National Natural Science Foundation of China (30771899) and in part by National Science and Technology Special Project of China (2008ZX10002-013).

References

Balagopal, A., Thomas, D.L., Thio, C.L., 2010. IL28B and the control of hepatitis C virus infection. Gastroenterology 139, 1865–1876.

Galmozzi, E., Del, M.B., Rametta, R., Dongiovanni, P., Fracanzani, A.L., Benedan, L., Borroni, V., Maggioni, P., Fargion, S., Valenti, L., 2011. A tetra-primer amplification refractory mutation system polymerase chain reaction for the evaluation of rs12979860 IL28B genotype. J. Viral Hepatitis 18, 628–630.

Ge, D., Fellay, J., Thompson, A.J., Simon, J.S., Shianna, K.V., Urban, T.J., Heinzen, E.L., Qiu, P., Bertelsen, A.H., Muir, A.J., Sulkowski, M., McHutchison, J.G., Goldstein, D.B., 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461, 399–401.

Ghany, M.G., Strader, D.B., Thomas, D.L., Seeff, L.B., 2009. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 49, 1335–1374.

Hoofnagle, J.H., Seeff, L.B., 2006. Peginterferon and ribavirin for chronic hepatitis C. N. Engl. J. Med. 355, 2444–2451.

Ito, K., Higami, K., Masaki, N., Sugiyama, M., Mukaide, M., Saito, H., Aoki, Y., Sato, Y., Imamura, M., Murata, K., Nomura, H., Hige, S., Adachi, H., Hino, K., Yatsuhashi, H., Orito, E., Kani, S., Tanaka, Y., Mizokami, M., 2011. The rs8099917 polymorphism, when determined by a suitable genotyping method, is a better predictor for response to pegylated alpha interferon/ribavirin therapy in Japanese patients than other single nucleotide polymorphisms associated with interleukin-28B. J. Clin. Microbiol. 49, 1853–1860.

Nakamoto, S., Kanda, T., Imazeki, F., Wu, S., Arai, M., Fujiwara, K., Yokosuka, O., 2011. Simple assay based on restriction fragment length polymorphism associated with IL28B in chronic hepatitis C patients. Scand. J. Gastroenterol. 46, 955–961.

Okayama, N., Fujimura, K., Nakamura, J., Suehiro, Y., Hamanaka, Y., Hinoda, Y., 2004. Evaluation of a new efficient procedure for single-nucleotide polymorphism genotyping: tetra-primer amplification refractory mutation system-polymerase chain reaction. Clin. Chem. Lab. Med. 42, 13–16.

Rauch, A., Kutalik, Z., Descombes, P., Cai, T., Di Iulio, J., Mueller, T., Bochud, M., Battegay, M., Bernasconi, E., Borovicka, J., Colombo, S., Cerny, A., Dufour, J.F., Furrer, H., Gunthard, H.F., Heim, M., Hirschel, B., Malinverni, R., Moradpour, D., Mullhaupt, B., Witteck, A., Beckmann, J.S., Berg, T., Bergmann, S., Negro, F., Telenti, A., Bochud, P.Y., 2010. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 138, 1338–1345, e1–e7.

S, L., 2001. Amplification-refractory mutation system (ARMS) analysis of point mutations. Curr Protoc Hum Genet. Chapter 9: Unit 9.8.

Sinn, D.H., Kim, Y.J., Lee, S.T., Gwak, G.Y., Choi, M.S., Lee, J.H., Koh, K.C., Yoo, B.C., Paik, S.W., 2011. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in Asian patients. J. Gastroenterol. Hepatol. 26, 1374–1379.

Suppiah, V., Moldovan, M., Ahlenstiel, G., Berg, T., Weltman, M., Abate, M.L., Bassendine, M., Spengler, U., Dore, G.J., Powell, E., Riordan, S., Sheridan, D., Smedile, A., Fragomeli, V., Muller, T., Bahlo, M., Stewart, G.J., Booth, D.R., George, J., 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. 41, 1100–1104.

Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., Nakagawa, M., Korenaga, M., Hino, K., Hige, S., Ito, Y., Mita, E., Tanaka, E., Mochida, S., Murawaki, Y., Honda, M., Sakai, A., Hiasa, Y., Nishiguchi, S., Koike, A., Sakaida, I., Imamura, M., Ito, K., Yano, K., Masaki, N., Sugauchi, F., Izumi, N., Tokunaga, K., Mizokami, M., 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. 41, 1105–1109.

Thompson, A.J., Muir, A.J., Sulkowski, M.S., Ge, D., Fellay, J., Shianna, K.V., Urban, T., Afdhal, N.H., Jacobson, I.M., Esteban, R., Poordad, F., Lawitz, E.J., McCone, J., Shiffman, M.L., Galler, G.W., Lee, W.M., Reindollar, R., King, J.W., Kwo, P.Y., Ghalib, R.H., Freilich, B., Nyberg, L.M., Zeuzem, S., Poynard, T., Vock, D.M., Pieper, K.S., Patel, K., Tillmann, H.L., Noviello, S., Koury, K., Pedicone, L.D., Brass, C.A., Albrecht, J.K., Goldstein, D.B., McHutchison, J.G., 2010. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology 139 (120–9), e18.

Ye, S., Dhillon, S., Ke, X., Collins, A.R., Day, I.N., 2001. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res. 29, 1–8.

Yu, M.L., Chuang, W.L., 2009. Treatment of chronic hepatitis C in Asia: when East meets West. J. Gastroenterol. Hepatol. 24, 336–345.