

respectively. Genotyping, the Invader method, of the four SNPs associated with IL28B in 416 patients in the second stage revealed that the four SNPs were not in LD in 6 cases (1.4%; Table 4). 410 (98.6%) of 416 cases were in LD for the four different SNPs. The second stage showed six different haplotypes (haplotypes 1-4, 6 and 7). Haplotypes 1-4 were detected in the first stage but haplotypes 6 and 7 were not. The distribution of haplotypes was that haplotypes 1, 2, 3 and 4 were found in 294 (70.7%), 110 (26.5%), 6 (1.4%) and 4 (1.0%) cases, respectively. In haplotype 6 (one case) rs11881222, rs8103142, rs12979860 and rs8099917 were AG, TT, CC, and TT, respectively. Haplotype 7 (one case) rs11881222, rs8103142, rs12979860 and rs8099917 were AA, TT, CT, and TG, respectively.

Response to PEG-IFN/RBV treatment in ten cases where the four SNPs associated with IL28B were not in LD

In 7 (case 1-7; 70%) of the 10 cases where the four SNPs were not in LD, the haplotype was such that rs11881222, rs8103142, rs12979860 and rs8099917 were AG, TC, CT, and TT, respectively (Table 5). In nine cases (case 1-9), rs8099917 was homozygous for the major allele, while one or more of the other SNPs were heterozygous. Eight (case 1-8) of these nine cases were viral responders who met the following criteria: HCV had disappeared during therapy or HCV-RNA had decreased more than 2 log copies/ml before 12 weeks after beginning of therapy, although some cases were under treatment or before determination of the final response to PEG-IFN/RBV. Case 9 was NVR due to poor adherence of PEG/IFN (< 50% dose), even

1 though rs8099917 was homozygous of major allele. The haplotype of case 9 showed that
2 rs11881222, rs8103142, rs12979860 and rs8099917 were AA, TT, CT, and TG,
3 respectively. NVR in Case 10 was reasonable from the genotypes of rs8099917 and
4 rs12979860, because they were heterozygous although rs11881222 and 8103142 were
5 homozygous for the major allele.

6

7 **DISCUSSION**

8 The relationship between SNPs associated with IL28B and the response to PEG-
9 IFN/RBV therapy for chronic hepatitis C were found by SNPs array, using GWAS
10 technology, by three different groups throughout the world, including our own, in 2009
11 (7, 19, 21). Following these reports, many studies have confirmed the association
12 between the response to PEG-IFN/RBV and SNPs associated with IL28B (14, 16).
13 Therefore, it is obvious that these SNPs may be valuable for predicting the response to
14 PEG-IFN/RBV therapy. Recently, it was reported that various SNPs were associated
15 with development of disease and response to therapy and correlated with adverse effects.
16 Several SNPs such as UGT1A1 polymorphism for the treatment with irinotecan (2, 17)
17 have already been exploited in clinical practice to avoid severe adverse effects. These
18 tailor-made therapies are expected to become more common in clinical practice in the
19 near future (9). The next step towards tailor-made therapy for PEG-IFN/RBV therapy
20 against chronic hepatitis C involved developing simple, accurate and inexpensive
21 methods to determine the genotype of SNPs and determining the best SNP where the
22 four SNPs associated with IL28B were not in LD, so that they may be applied in clinical

1 practice.

2 Genotyping of IL28B SNPs is quite different from other SNPs, because the sequence
 3 of IL28B is very similar to those of IL28A, IL29 and an additional homologous
 4 sequence upstream of IL28B (Figure 2). We had to design primers and probes for each
 5 method to distinguish IL28B specifically. We think that the results in this paper are
 6 especially applicable to IL28B genotyping. In this study, only HRM failed to determine
 7 the genotype of SNPs associated with IL28B. The reason HRM failed more frequently
 8 than the other genotyping methods is attributable to the characteristics of this specific
 9 method. Because HRM determines the genotype of each SNP by distinguish the melting
 10 curve of an amplicon of around 200bp, it may tend to be influenced by another SNP. As
 11 a matter of fact, minor SNPs around rs8099917 were found in cases of genotyping
 12 failure by HRM (data not shown). Although this specific characteristic of the HRM
 13 method is useful for detecting novel mutations or SNPs, it is not suitable for determining
 14 the genotype of SNPs associated with IL28B.

15 Direct sequencing erroneously reported the T/G genotype as T/T for the rs8099917
 16 polymorphism. We found that the cause of this genotyping error was a novel rare SNP in
 17 the forward primer binding site used for amplification and direct sequencing (data on
 18 file). Because this novel SNP was not registered as a SNP in the NCBI database, the
 19 primer was designed at this site. Since the novel SNP correlated with the rs8099917
 20 polymorphism in LD, adenine for the novel SNP is present on the same allele as guanine
 21 in the rs8099917 polymorphism. Therefore, the forward PCR primer
 22 (AAGTAACACTTGTTCTTGTAAAAGATTCC) could not anneal to the binding site,

which was changed from guanine (G) to adenine (A) at the underlined nucleotide position, only the allele which has T at the rs8099917 was amplified the genotype was determined as T/T. Rare sequence variations not registered in the database, might be present in the primer binding sites for amplification, and be the cause of erroneous direct sequencing. Ikegawa et al. reported that annealing efficiency in direct sequencing led to the mistyping of a SNP(10). Although our results in this paper are especially applicable to IL28B genotyping, it should be recognized that allele-dependent PCR amplification and erroneous typing can occur when SNPs are genotyped using a PCR-based approach. Should SNPs associated with IL28B be found not to be in LD, it would be preferable to confirm the genotype by another method.

In ten cases, four SNPs associated with IL28B were not in LD. In seven (70%) of the ten cases, the haplotype showed that rs11881222, rs8103142, rs12979860 and rs8099917 were AG, TC, CT, and TT, respectively. Only the rs8099917 polymorphism differed frequently from the other three SNPs. The reason for the high frequency of this haplotype is thought to be attributable to the location of these SNPs. The location of rs8099917 is downstream and quite far from the two SNPs (rs11881222 and rs8103142) in the IL28B gene (Figure 2). The SNPs rs11881222 and rs8103142 were almost perfectly in LD, because they are located close to each other.

It is well described that homozygosity for the major allele of SNPs associated with IL28B is correlated with a better response to PEG-IFN/RBV treatment and minor allele-positive patients are poor responders. However, the response to PEG-IFN/RBV remains unknown when several SNPs associated with IL28B are not in LD. Because cases in

1 which the SNPs are not in LD are quite rare, it was thought to be difficult to study such
2 cases. In this study, 10 (1.4%) of 708 patients showed haplotypes in which the four
3 SNPs were not in LD. We focused on the response to PEG-IFN/RBV therapy in these 10
4 cases (Table 5). We evaluated the response to PEG-IFN/RBV treatment from the view
5 point of virological response, because some patients had not completed their PEG-
6 IFN/RBV treatment (case 3 was before determination for the final response after
7 finishing the treatment and cases 4-6 were under treatment).

8 Thomas et al. reported that allele frequencies for the rs12979860 were vary among
9 racial and ethnic groups (23). Indeed, the observation that the major allele is less
10 frequent among individuals of African descent relative to those of European descent
11 might explain, the observed discrepancy in the frequency of viral clearance in these two
12 ethnic groups, where clearance occurs in 36.4% of HCV infections in individuals of non-
13 African ancestry, but only in 9.3% of infections in individuals of African ancestry (22).
14 We have recruited only Japanese chronic hepatitis C patients for this study. Since the
15 distribution of haplotype and response to PEG-IFN/RBV treatment should be vary
16 among populations, further study will be necessary for any other populations except
17 Japanese.

18 We have shown that the rs8099917 polymorphism determined by Invader assay should
19 be the best predictor of the response to PEG-IFN/RBV in Japanese chronic hepatitis C
20 patients.

21

22 **ACKNOWLEDGEMENT**

This study was supported by grants (22-302 and 21-113) from National Center for Global Health and Medicine in Japan.

FIGURE LEGENDS

Figure 1. Schema for flow chart of the examinations

Figure 2. Location of interferon lambda genes and the four SNPs (rs11881222, rs8103142, rs12979860 and rs8099917) associated with IL28B

Figure 3. The nucleotide sequence around rs8099917 is shown. Primers and probes for four different methods (Sequence: direct sequencing, HRM: high-resolution melting analysis, HP: Hybridization probe, Invader: InvaderPlus assay) to determine rs8099917 polymorphism are shown. F: forward primer. R: reverse primer.

Figure 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence: direct sequencing, HRM: high-resolution melting analysis, HP: Hybridization probe, Invader: InvaderPlus assay, TaqMan: TaqMan assay) to determine rs11881222 and rs8103142 polymorphisms are shown. F: forward primer. R: reverse primer.

Figure 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for

- 1 four different methods (Sequence: direct sequencing, HRM: high-resolution melting
- 2 analysis, HP: Hybridization probe, Invader: InvaderPlus assay) to determine rs12979860
- 3 are shown. F: forward primer. R: reverse primer.

1. 2004. Global burden of disease (GBD) for hepatitis C. *J Clin Pharmacol* **44**:20-9.
2. Ando, Y., H. Saka, M. Ando, T. Sawa, K. Muro, H. Ueoka, A. Yokoyama, S. Saitoh, K. Shimokata, and Y. Hasegawa. 2000. Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* **60**:6921-6.
3. Bruno, S., C. Camma, V. Di Marco, M. Rumi, M. Vinci, M. Camozzi, C. Rebucci, D. Di Bona, M. Colombo, A. Craxi, M. U. Mondelli, and G. Pinzello. 2004. Peginterferon alfa-2b plus ribavirin for naive patients with genotype 1 chronic hepatitis C: a randomized controlled trial. *J Hepatol* **41**:474-81.
4. Fried, M. W., M. L. Shiffman, K. R. Reddy, C. Smith, G. Marinos, F. L. Goncalves, Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, and J. Yu. 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**:975-82.
5. Froehlich, T., and O. Geulen. 2008. Hybridization probe pairs and single-labeled probes: an alternative approach for genotyping and quantification. *Methods Mol Biol* **429**:117-33.
6. Garritano, S., F. Gemignani, C. Voegelé, T. Nguyen-Dumont, F. Le Calvez-Kelm, D. De Silva, F. Lesueur, S. Landi, and S. V. Tavtigian. 2009. Determining the effectiveness of High Resolution Melting analysis for SNP genotyping and mutation scanning at the TP53 locus. *BMC Genet* **10**:5.
7. Ge, D., J. Fellay, A. J. Thompson, J. S. Simon, K. V. Shianna, T. J. Urban, E. L. Heinzen, P. Qiu, A. H. Bertelsen, A. J. Muir, M. Sulkowski, J. G. McHutchison, and D. B. Goldstein. 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461**:399-401.
8. Hadziyannis, S. J., H. Sette, Jr., T. R. Morgan, V. Balan, M. Diago, P. Marcellin, G. Ramadori, H. Bodenheimer, Jr., D. Bernstein, M. Rizzetto, S. Zeuzem, P. J. Pockros, A. Lin, and A. M. Ackrill. 2004. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* **140**:346-55.
9. Iadonato, S. P., and M. G. Katze. 2009. Genomics: Hepatitis C virus gets personal. *Nature* **461**:357-8.
10. Ikegawa, S., A. Mabuchi, M. Ogawa, and T. Ikeda. 2002. Allele-specific PCR amplification due to sequence identity between a PCR primer and an amplicon: is direct sequencing so reliable? *Hum Genet* **110**:606-8.
11. Lyamichev, V., A. L. Mast, J. G. Hall, J. R. Prudent, M. W. Kaiser, T. Takova, R. W. Kwiatkowski, T. J. Sander, M. de Arruda, D. A. Arco, B. P. Neri, and M. A. Brow. 1999. Polymorphism identification and quantitative detection of genomic DNA by invasive cleavage of oligonucleotide probes. *Nat Biotechnol* **17**:292-6.
12. Lyamichev, V. I., M. W. Kaiser, N. E. Lyamicheva, A. V. Vologodskii, J. G. Hall, W. P. Ma, H. T. Allawi, and B. P. Neri. 2000. Experimental and theoretical analysis of the invasive signal amplification reaction. *Biochemistry* **39**:9523-32.

13. Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling, and J. K. Albrecht. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**:958-65.
14. Montes-Cano, M. A., J. R. Garcia-Lozano, C. Abad-Molina, M. Romero-Gomez, N. Barroso, J. Aguilar-Reina, A. Nunez-Roldan, and M. F. Gonzalez-Escribano. Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. *Hepatology* **52**:33-7.
15. Nguyen-Dumont, T., F. L. Calvez-Kelm, N. Forey, S. McKay-Chopin, S. Garritano, L. Gioia-Patricola, D. De Silva, R. Weigel, S. Sangrajang, F. Lesueur, and S. V. Tavtigian. 2009. Description and validation of high-throughput simultaneous genotyping and mutation scanning by high-resolution melting curve analysis. *Hum Mutat* **30**:884-90.
16. Rauch, A., Z. Kutalik, P. Descombes, T. Cai, J. Di Iulio, T. Mueller, M. Bochud, M. Battegay, E. Bernasconi, J. Borovicka, S. Colombo, A. Cerny, J. F. Dufour, H. Furrer, H. F. Gunthard, M. Heim, B. Hirschel, R. Malinverni, D. Moradpour, B. Mullhaupt, A. Witteck, J. S. Beckmann, T. Berg, S. Bergmann, F. Negro, A. Telenti, and P. Y. Bochud. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. *Gastroenterology* **138**:1338-45, 1345 e1-7.
17. Rouits, E., M. Boisdron-Celle, A. Dumont, O. Guerin, A. Morel, and E. Gamelin. 2004. Relevance of different UGT1A1 polymorphisms in irinotecan-induced toxicity: a molecular and clinical study of 75 patients. *Clin Cancer Res* **10**:5151-9.
18. Stamer, U. M., B. Bayerer, S. Wolf, A. Hoefft, and F. Stuber. 2002. Rapid and reliable method for cytochrome P450 2D6 genotyping. *Clin Chem* **48**:1412-7.
19. Suppiah, V., M. Moldovan, G. Ahlenstiel, T. Berg, M. Weltman, M. L. Abate, M. Bassendine, U. Spengler, G. J. Dore, E. Powell, S. Riordan, D. Sheridan, A. Smedile, V. Fragomeli, T. Muller, M. Bahlo, G. J. Stewart, D. R. Booth, and J. George. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* **41**:1100-4.
20. Tadokoro, K., T. Yamaguchi, K. Kawamura, H. Shimizu, T. Egashira, M. Minabe, T. Yoshino, and H. Oguchi. Rapid quantification of periodontitis-related bacteria using a novel modification of Invader PLUS technologies. *Microbiol Res* **165**:43-9.
21. Tanaka, Y., N. Nishida, M. Sugiyama, M. Kurosaki, K. Matsuura, N. Sakamoto, M. Nakagawa, M. Korenaga, K. Hino, S. Hige, Y. Ito, E. Mita, E. Tanaka, S. Mochida, Y. Murawaki, M. Honda, A. Sakai, Y. Hiasa, S. Nishiguchi, A. Koike, I. Sakaida, M. Imamura, K. Ito, K. Yano, N. Masaki, F. Sugauchi, N. Izumi, K. Tokunaga, and M. Mizokami. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin

- therapy for chronic hepatitis C. *Nat Genet* **41**:1105-9.
22. **Thomas, D. L., J. Astemborski, R. M. Rai, F. A. Anania, M. Schaeffer, N. Galai, K. Nolt, K. E. Nelson, S. A. Strathdee, L. Johnson, O. Laeyendecker, J. Boitnott, L. E. Wilson, and D. Vlahov.** 2000. The natural history of hepatitis C virus infection: host, viral, and environmental factors. *JAMA* **284**:450-6.
 23. **Thomas, D. L., C. L. Thio, M. P. Martin, Y. Qi, D. Ge, C. O'Huigin, J. Kidd, K. Kidd, S. I. Khakoo, G. Alexander, J. J. Goedert, G. D. Kirk, S. M. Donfield, H. R. Rosen, L. H. Tobler, M. P. Busch, J. G. McHutchison, D. B. Goldstein, and M. Carrington.** 2009. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461**:798-801.
 24. **Whitehall, V., K. Tran, A. Umapathy, F. Grieu, C. Hewitt, T. J. Evans, T. Ismail, W. Q. Li, P. Collins, P. Ravetto, B. Leggett, M. Salto-Tellez, R. Soong, S. Fox, R. J. Scott, A. Dobrovic, and B. Iacopetta.** 2009. A multicenter blinded study to evaluate KRAS mutation testing methodologies in the clinical setting. *J Mol Diagn* **11**:543-52.
 25. **Younossi, Z., J. Kallman, and J. Kincaid.** 2007. The effects of HCV infection and management on health-related quality of life. *Hepatology* **45**:806-16.

Table 1. Characteristics of the nominated patients

	1st stage (n=292)	2nd stage (n=416)
Age	57.2±10.2	56.6±10.9
Gender (Male/Female)	145/147	194/222
Institutions		
#1	18 (6.2)	0 (0)
#2	178 (61.0)	0 (0)
#3	57 (19.5)	0 (0)
#4	39 (13.3)	0 (0)
#5	0 (0)	249 (59.9)
#6	0 (0)	94 (22.6)
#7	0 (0)	52 (12.5)
#8	0 (0)	21 (5.0)

Institutions: #1 The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, #2 The Center for Liver Diseases, Shin-Kokura Hospital, Kitakyushu, #3 Tonami General Hospital, Tonami, #4 Department of Internal Medicine, Virology and Liver Unit, Hokkaido University Graduate School of Medicine, Sapporo, #5 Clinical Research Center, NHO Nagasaki Medical Center, Nagasaki, #6 Nagoya City University Graduate School of Medical Sciences, Nagoya, #7 Department of Gastroenterology and Hepatology, Nagoya Daini Red Cross Hospital, #8 Division of Gastroenterology, Department of Medicine, Kawasaki Medical School, Okayama

Table 2. Four SNPs associated with IL28B were determined by four different methods

SNPs	Genotype	Sequence	HP	Invader	TaqMan
rs11881222	AA (%)	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	AG (%)	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	GG (%)	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
	Consistency (%)	100%			
rs8103142	TT (%)	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	TC (%)	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	CC (%)	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
	Consistency (%)	100%			
rs12979860	CC (%)	198 (69.0)	198 (69.0)	198 (69.0)	198 (69.0)
	CT (%)	85 (29.6)	85 (29.6)	85 (29.6)	85 (29.6)
	TT (%)	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
	Consistency (%)	100%			
rs8099917	TT (%)	204 (71.1)	202 (70.4)	202 (70.4)	202 (70.4)
	TG (%)	79 (27.5)	81 (28.2)	81 (28.2)	81 (28.2)
	GG (%)	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
	Consistency (%)	99.3%			

Table 3. Genotyping rs8099917 by direct sequencing was inconsistent with three other methods in two cases.

rs8099917				
	Sequence	HP	Invader	TaqMan
Case 1	T/T	T/G	T/G	T/G
Case 2	T/T	T/G	T/G	T/G

Table 4. Distribution of Haplotype among four SNPs associated with IL28B in stages 1 and 2

SNPs		rs11881222	rs8103142	rs12979860	rs8099917	n (%)
stage 1	Haplotype 1	AA	TT	CC	TT	198 (69.5%)
	Haplotype 2	AG	TC	CT	TG	79 (27.7%)
	Haplotype 3	GG	CC	TT	GG	4 (1.4%)
	Haplotype 4	AG	TC	CT	TT	3 (1.0%)
	Haplotype 5	AA	TT	CT	TT	1 (0.4%)
SNPs		rs11881222	rs8103142	rs12979860	rs8099917	n (%)
stage 2	Haplotype 1	AA	TT	CC	TT	294 (70.7%)
	Haplotype 2	AG	TC	CT	TG	110 (26.5%)
	Haplotype 3	GG	CC	TT	GG	6 (1.4%)
	Haplotype 4	AG	TC	CT	TT	4 (1.0%)
	Haplotype 6	AG	TT	CC	TT	1 (0.2%)
	Haplotype 7	AA	TT	CT	TG	1 (0.2%)

Table 5. Clinical characteristics of ten cases in which the SNPs associated with IL28B were not in LD

Case	SNPs of IL28B				Age	Gender	Treatment	Genotype	Viral Titer	Final Response to PEG/IFN	VR or NVR	Period of Disappearance of HCV
	rs11881222	rs8103142	rs12979860	rs8099917								
1	A/G	T/C	C/T	T/T	64	Female	PegIFN/RBV	1b	6.5	TR	VR	4W
2	A/G	T/C	C/T	T/T	72	Male	PegIFN/RBV	1b	2.9	SVR	VR	4W
3	A/G	T/C	C/T	T/T	64	Male	PegIFN/RBV	1b	7	ND*	VR	8W
4	A/G	T/C	C/T	T/T	51	Female	PegIFN/RBV	1b	7.2	under treatment	VR	3.6 log down after 12W
5	A/G	T/C	C/T	T/T	60	Female	PegIFN/RBV	2	5.8	under treatment	VR	12W
6	A/G	T/C	C/T	T/T	56	Female	PegIFN/RBV	1b	5.9	under treatment	VR	2.0 log down after 2W
7	A/G	T/C	C/T	T/T	62	Male	PegIFN/RBV	1b	5.4	SVR	VR	4W
8	A/G	T/T	C/C	T/T	58	Male	PegIFN/RBV	1b	6.2	TR	VR	12W
9	A/A	T/T	C/T	T/T	68	Male	PegIFN/RBV	1b	7	NVR	NVR	(-)
10	A/A	T/T	C/T	T/G	48	Female	PegIFN/RBV	1b	6	NVR	NVR	(-)

*ND : The final response to PEG/IFN was not determined in this patient, because six months had not passed after the end of treatment.

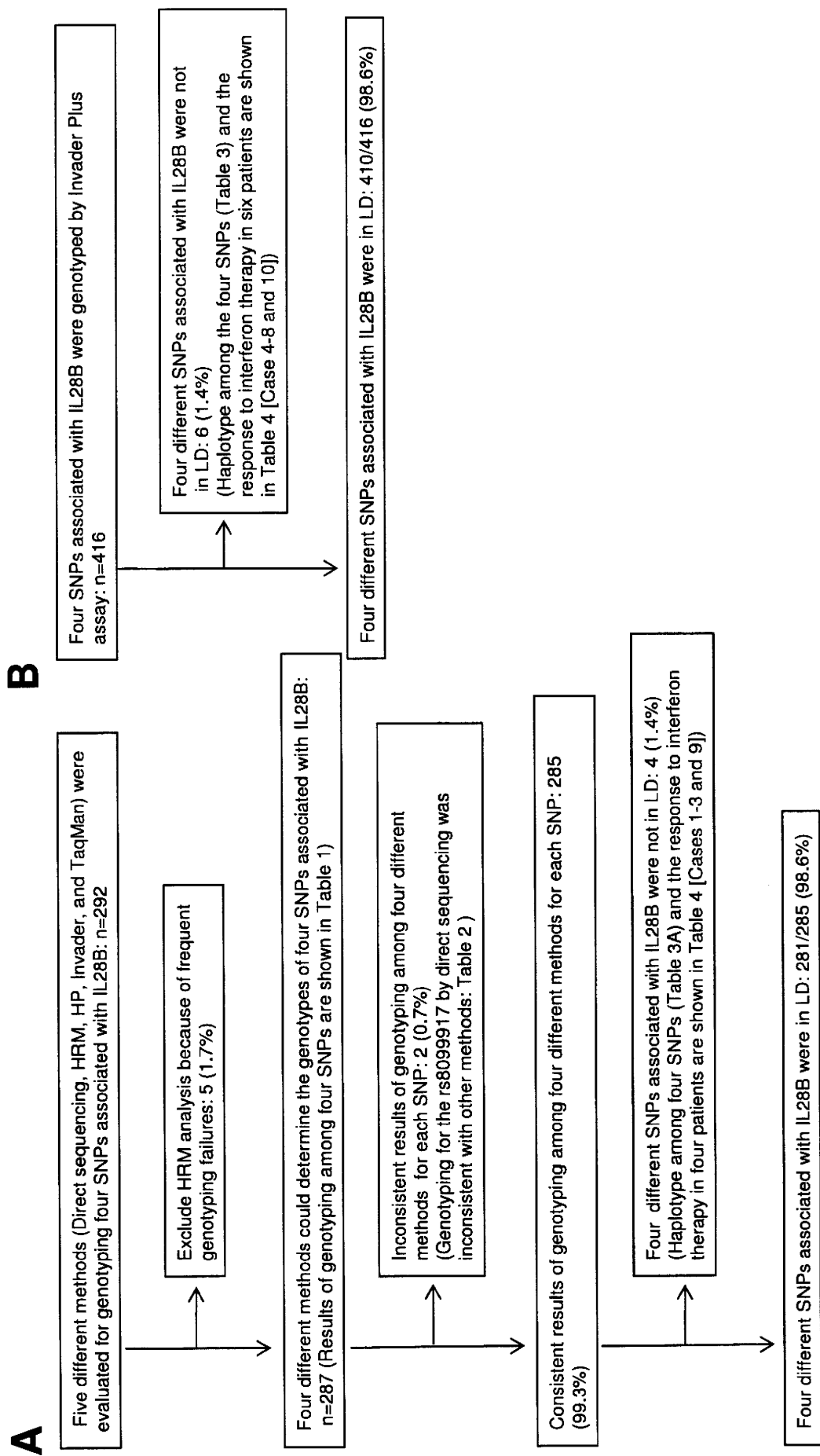


Figure 1

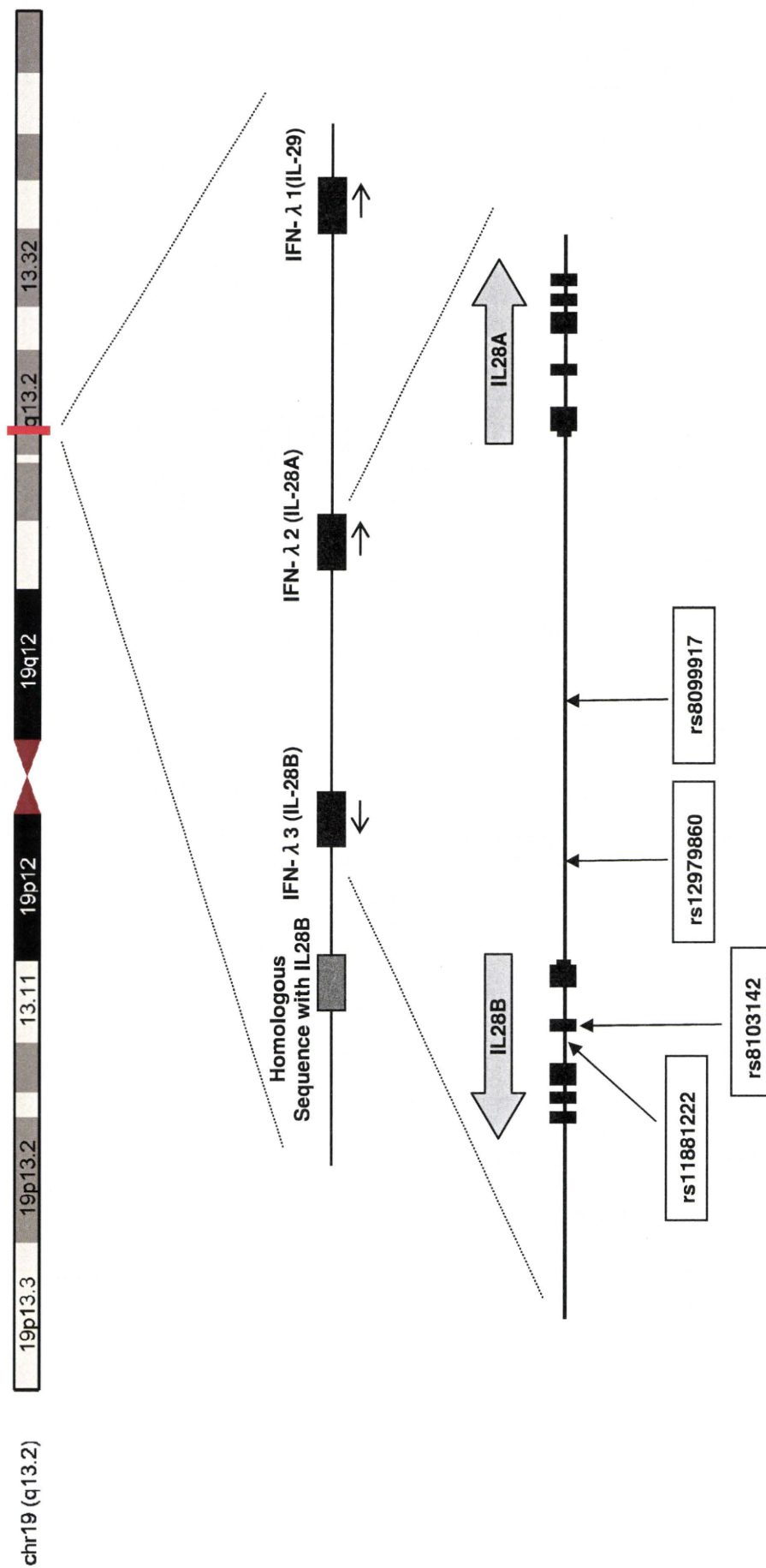


Figure 2

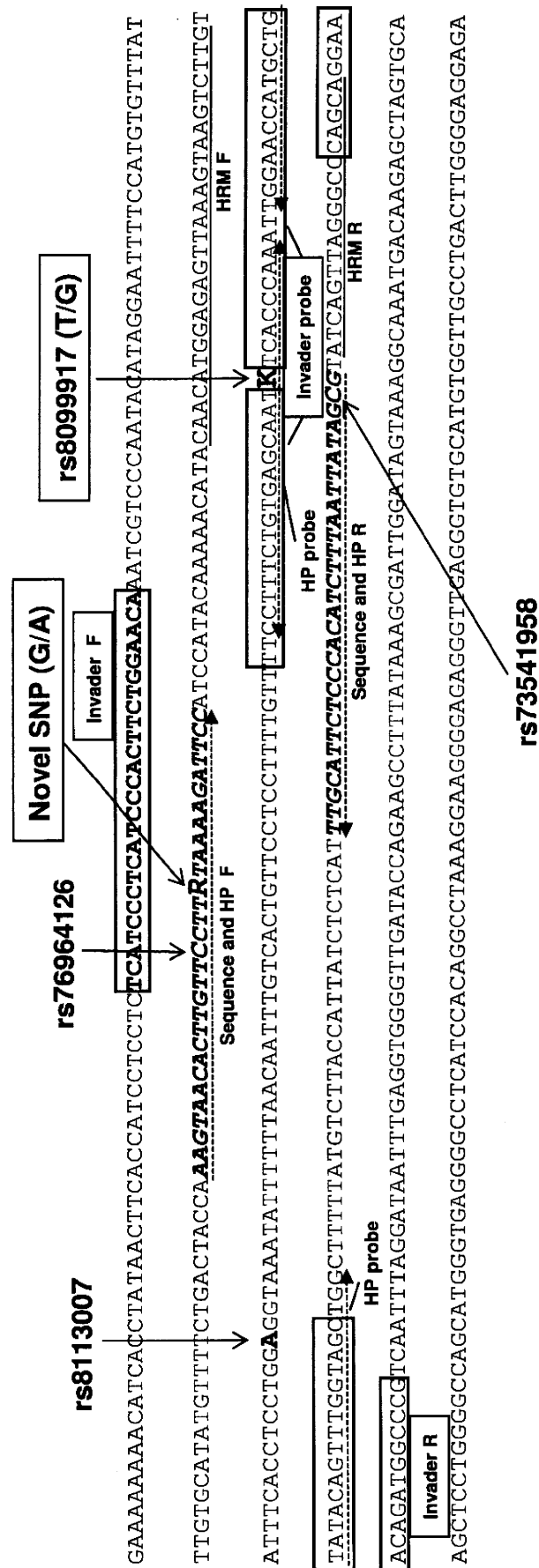


Figure 3

