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

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

DISCUSSION

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

practice.

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

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

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

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

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

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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

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

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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.

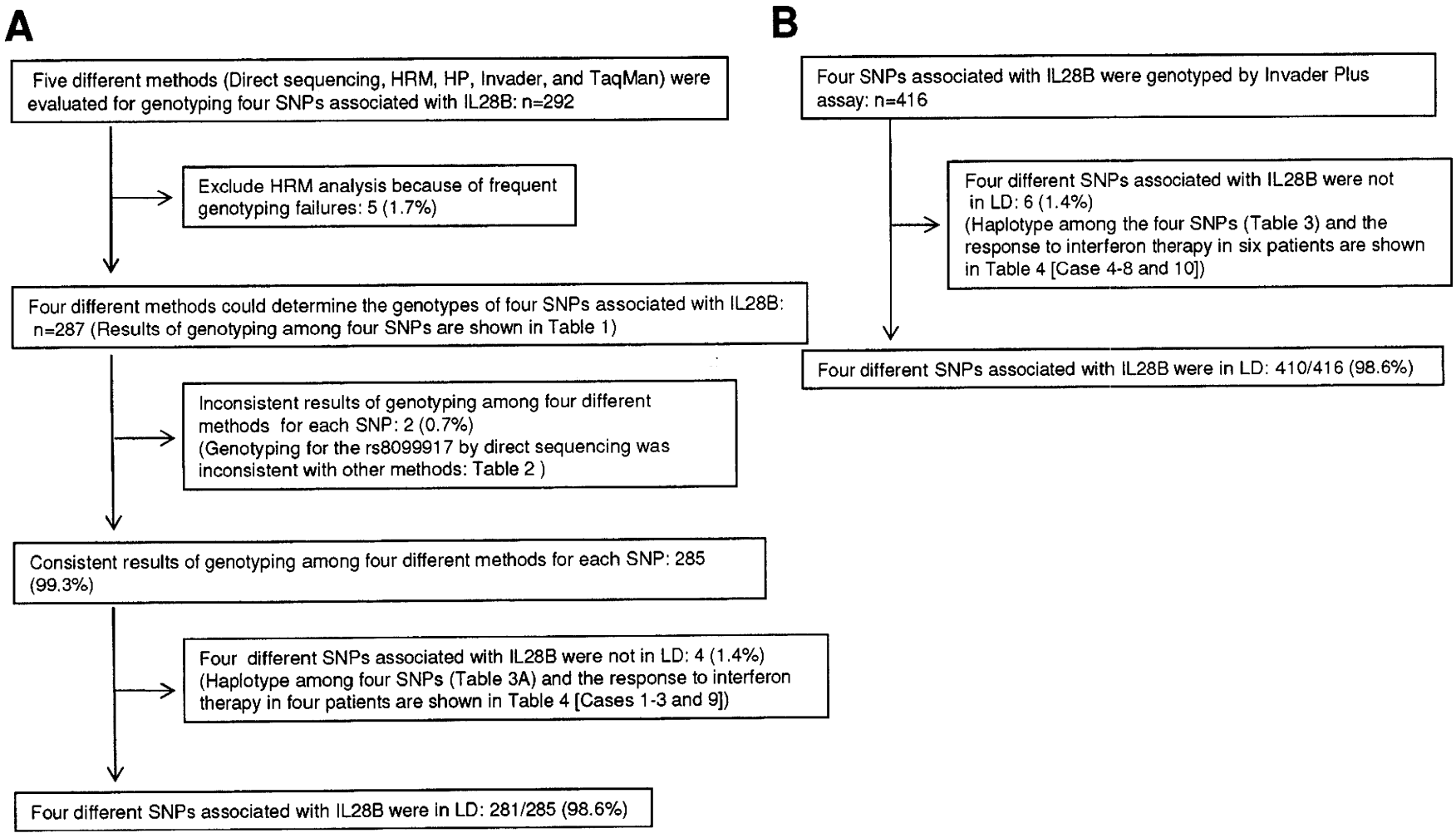
A
Five different methods (Direct sequencing, HRM, HP, Invader, and TaqMan) were evaluated for genotyping four SNPs associated with IL28B: n=292
Exclude HRM analysis because of frequent genotyping failures: 5 (1.7%)
Four different methods could determine the genotypes of four SNPs associated with IL28B: n=287 (Results of genotyping among four SNPs are shown in Table 1)
Inconsistent results of genotyping among four different methods for each SNP: 2 (0.7%)
(Genotyping for the rs8099917 by direct sequencing was inconsistent with other methods: Table 2)
Consistent results of genotyping among four different methods for each SNP: 285 (99.3%)
Four different SNPs associated with IL28B were not in LD: 4 (1.4%)
(Haplotype among four SNPs (Table 3A) and the response to interferon therapy in four patients are shown in Table 4 [Cases 1-3 and 9])
Four different SNPs associated with IL28B were in LD: 281/285 (98.6%)
B
Four SNPs associated with IL28B were genotyped by Invader Plus assay: n=416
Four different SNPs associated with IL28B were not in LD: 6 (1.4%)
(Haplotype among the four SNPs (Table 3) and the response to interferon therapy in six patients are shown in Table 4 [Case 4-8 and 10])
Four different SNPs associated with IL28B were in LD: 410/416 (98.6%)

Figure 1

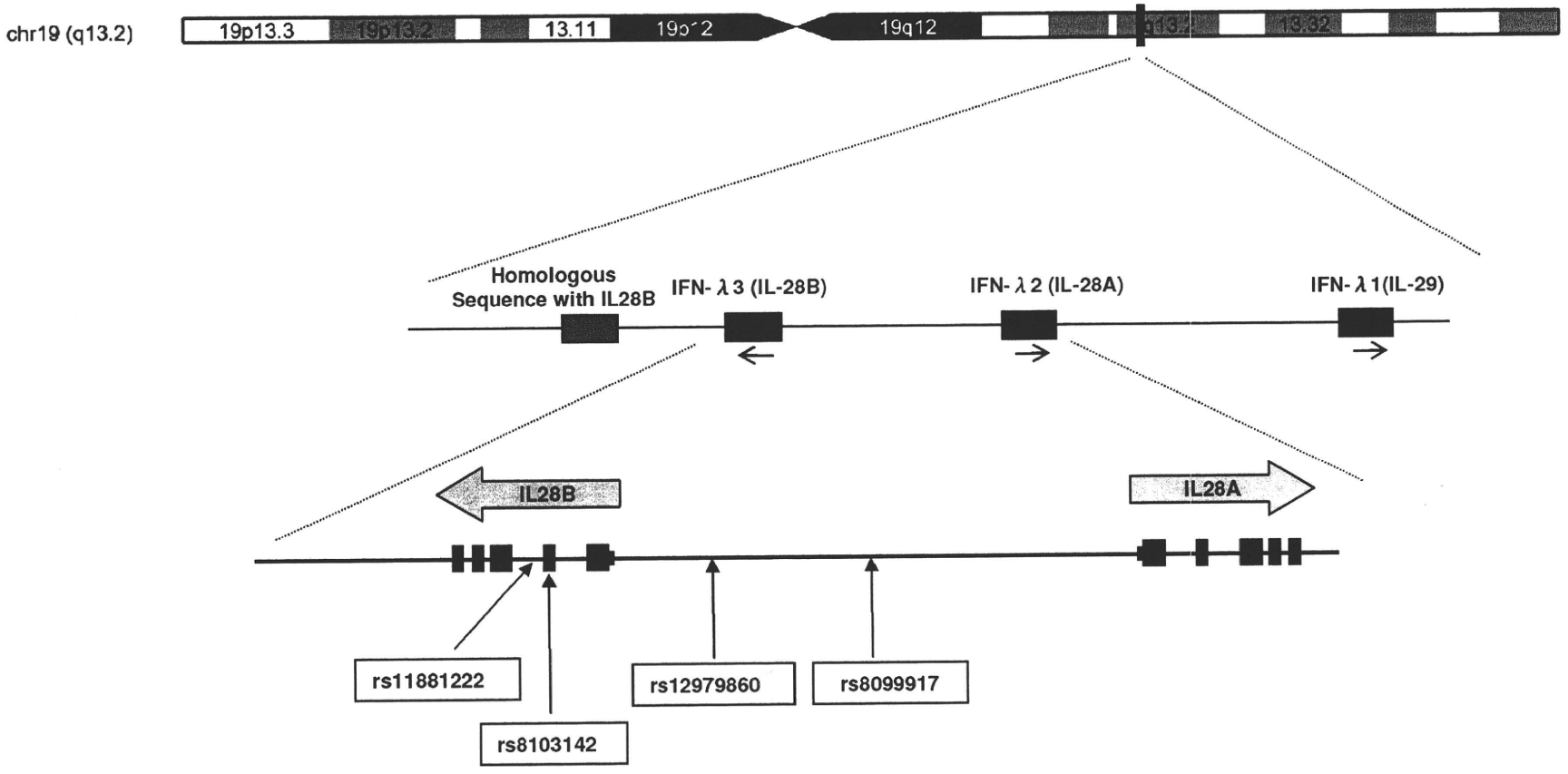
chr19 (q13.2)
19p13.3
13.11
19q12
Homologous
Sequence with IL28B
IFN- λ 3 (IL-28B)
IFN- λ 2 (IL-28A)
IFN- λ 1(IL-29)
IL28B
IL28A
rs11881222
rs8103142
rs12979860
rs8099917

Figure 2

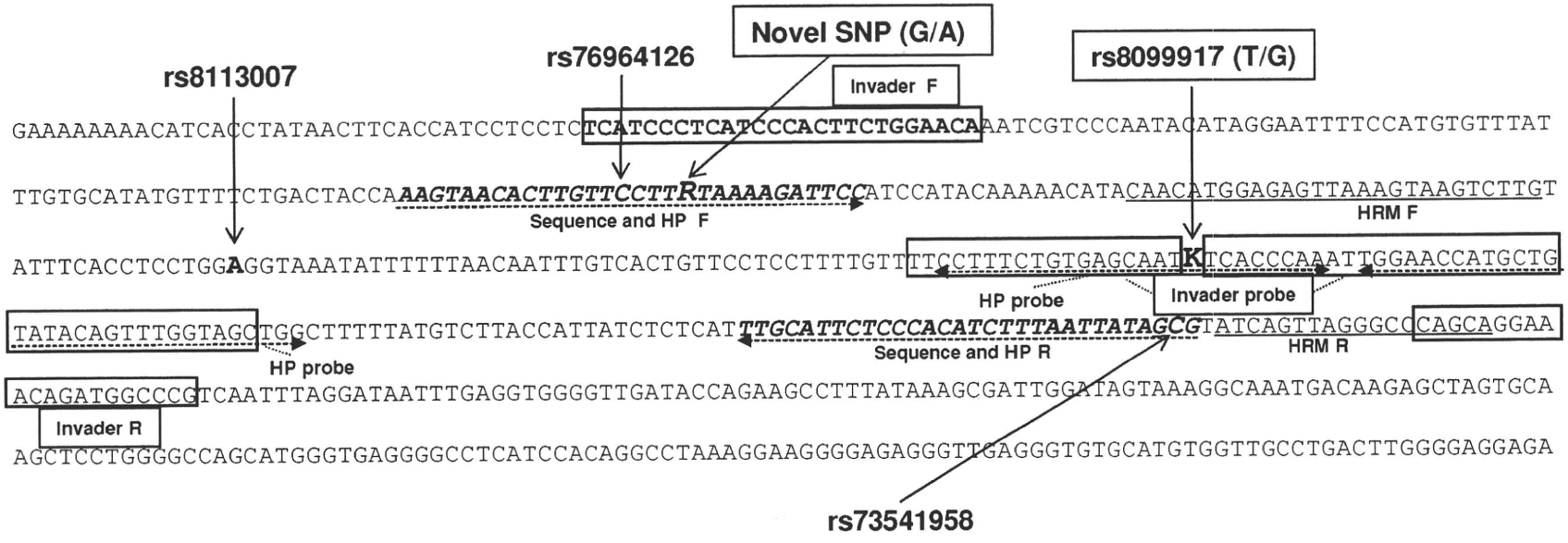
Novel SNP (G/A)
rs8113007
rs76964126
rs8099917 (T/G)
Invader F
GAAAAAAAACATCACCTATAACTTCACCATCCTCCTCTCATCCCTCATCCCACTTCTGGAACAAATCGTCCCAATACATAGGAATTTTCCATGTGTTTAT
TTGTGCATATGTTTTCTGACTACCAAAGTAACACTTGTTCCTTRTAAAAGATTCCCATCCATACAAAAACATACAACATGGAGAGTTAAAGTAAGTCTTGT
Sequence and HP F
HRM F
ATTTCACCTCCTGGAGGTAAATATTTTTTAACAATTTGTCACTGTTCCTCCTTTTGTTTTCCTTTCTGTGAGCAATKTCACCCAAATTGGAACCATGCTG
HP probe
Invader probe
TATACAGTTTGGTAGCTGGCTTTTTATGTCTTACCATTATCTCTCATTTGCATTCTCCCACATCTTTAATTATAGCGTATCAGTTAGGGCCCAGCAGGAA
HP probe
Sequence and HP R
HRM R
ACAGATGGCCCGTCAATTTAGGATAATTTGAGGTGGGGTTGATACCAGAAGCCTTTATAAAGCGATTGGATAGTAAAGGCAAATGACAAGAGCTAGTGCA
Invader R
AGCTCCTGGGGCCAGCATGGGTGAGGGGCCTCATCCACAGGCCTAAAGGAAGGGGAGAGGGTTGAGGGTGTGCATGTGGTTGCCTGACTTGGGGAGGAGA
rs73541958

Figure 3

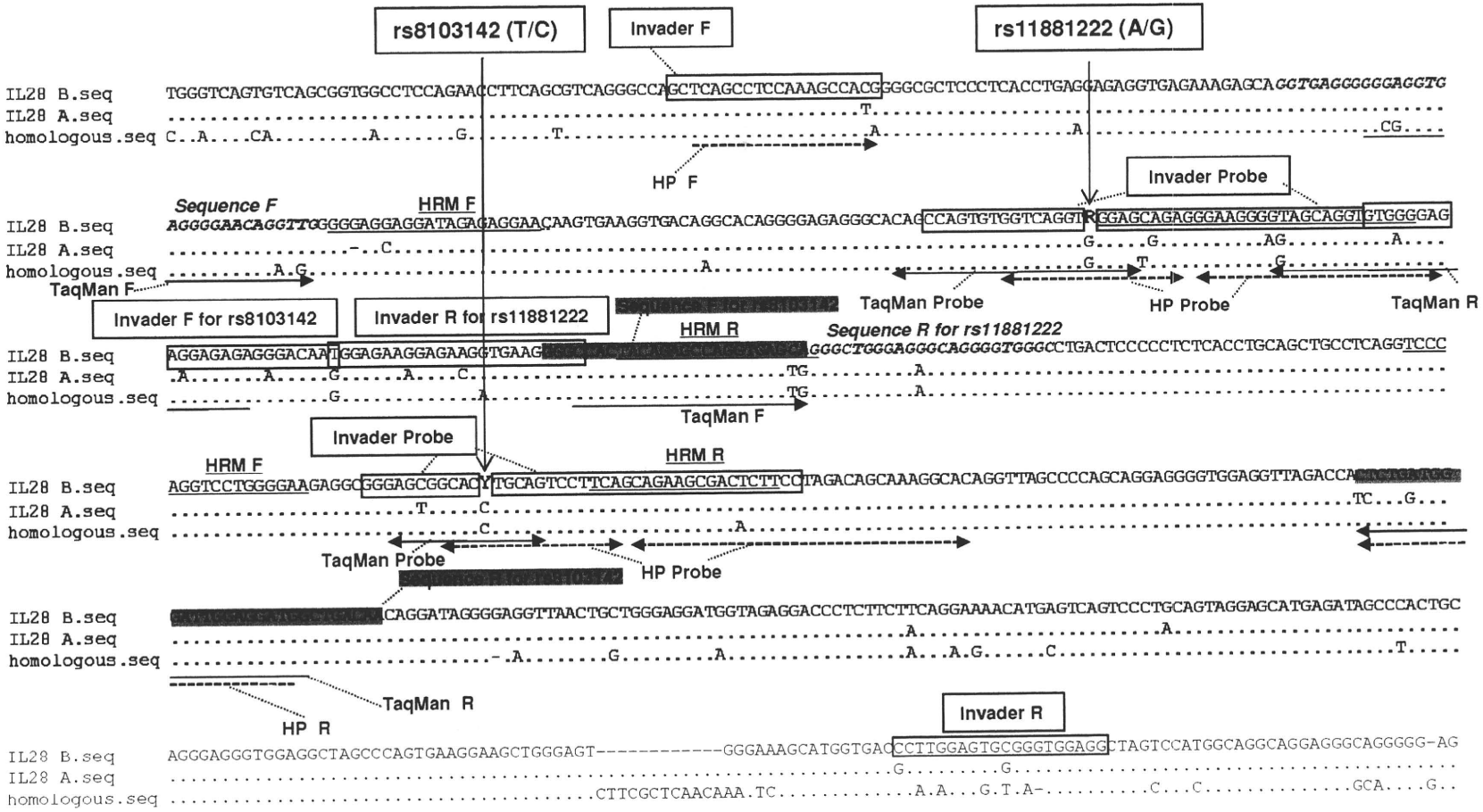
rs8103142 (T/C)
Invader F
rs11881222 (A/G)
IL28 B.seq
IL28 A.seq
homologous.seq
HP F
Invader Probe
Sequence F
HRM F
TaqMan F
Invader F for rs8103142
Invader R for rs11881222
HRM R
Sequence R for rs11881222
TaqMan Probe
HP Probe
TaqMan R
Invader Probe
HRM F
HRM R
TaqMan Probe
HP Probe
TaqMan R
HP R
Invader R

Figure 4

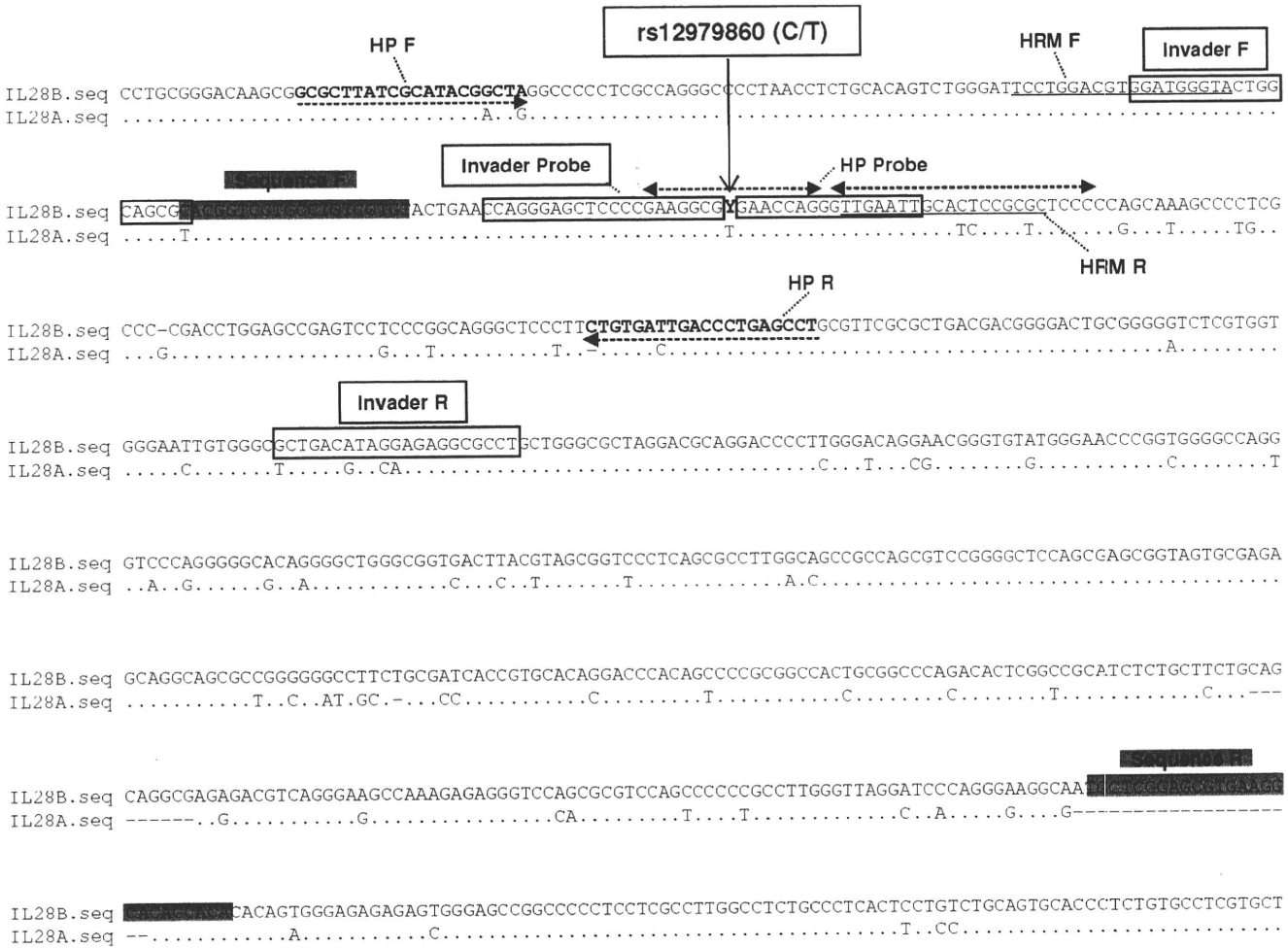
rs12979860 (C/T)
HP F
HRM F
Invader F
Invader Probe
HP Probe
HRM R
HP R
Invader R
IL28B.seq CCTGCGGGACAAGCGGCGCTTATCGCATACGGCTAGGCCCCCTCGCCAGGGCCCCTAACCTCTGCACAGTCTGGGATTCCTGGACGTGGATGGGTACTGG
IL28A.seq .....A..G.....
IL28B.seq CAGCG[redacted]ACTGAACCAGGGAGCTCCCCGAAGGCGYGAACCAGGGTTGAATTGCACTCCGCGCTCCCCCAGCAAAGCCCCTCG
IL28A.seq .....T.....T.....TC....T.....G...T.....TG..
IL28B.seq CCC-CGACCTGGAGCCGAGTCCTCCCGGCAGGGCTCCCTTCTGTGATTGACCCTGAGCCTGCGTTCGCGCTGACGACGGGGACTGCGGGGGTCTCGTGGT
IL28A.seq ...G.....G...T.....T..-.....C.....A.....
IL28B.seq GGGAATTGTGGGGCTGACATAGGAGAGGCGCCTGCTGGGCGCTAGGACGCAGGACCCCTTGGGACAGGAACGGGTGTATGGGAACCCGGTGGGGCCAGG
IL28A.seq .....C.....T.....G..CA.....C...T...CG.....G.....C.....T
IL28B.seq GTCCCAGGGGGCACAGGGGCTGGGCGGTGACTTACGTAGCGGTCCCTCAGCGCCTTGGCAGCCGCCAGCGTCCGGGGCTCCAGCGAGCGGTAGTGCGAGA
IL28A.seq ..A..G.....G..A.....C...C..T.....T.....A.C.....
IL28B.seq GCAGGCAGCGCCGGGGGGCCTTCTGCGATCACCGTGCACAGGACCCACAGCCCCGCGGCCACTGCGGCCCAGACACTCGGCCGCATCTCTGCTTCTGCAG
IL28A.seq .....T..C..AT.GC.-...CC.....C.....T.....C.....C.....T.....C...---
IL28B.seq CAGGCGAGAGACGTCAGGGAAGCCAAAGAGAGGGTCCAGCGCGTCCAGCCCCCCGCCTTGGGTTAGGATCCCAGGGAAGGCAA[redacted]
IL28A.seq ------..G.....G.....CA.....T....T.....C..A.....G....G----------
IL28B.seq [redacted]CACAGTGGGAGAGAGAGTGGGAGCCGGCCCCCTCCTCGCCTTGGCCTCTGCCCTCACTCCTGTCTGCAGTGCACCCTCTGTGCCTCGTGCT
IL28A.seq --.....A.....C.....T..CC.....

Figure 5