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ORIGINAL ARTICLE—ALIMENTARY TRACT

Characteristics of Japanese inflammatory bowel disease susceptibility loci

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Abstract

Background There are substantial differences in inflammatory bowel disease (IBD) genetics depending on the populations examined. We aimed to identify Japanese population-specific or true culprit susceptibility genes through a meta-analysis of past genetic studies of Japanese IBD.

Methods For this study, we reviewed 2,703 articles. The review process consisted of three screening stages: we initially searched for relevant studies and then relevant single nucleotide polymorphisms (SNPs). Finally, we adjusted them for the meta-analysis. To maximize our chances of analysis, we introduced proxy SNPs during the first stage. To minimize publication bias, no significant SNPs and solitary SNPs without pairs were combined to be reconsidered during the third stage. Additionally, two SNPs were newly genotyped. Finally, we conducted a meta-analysis of 37 published studies in 50 SNPs located at 22 loci corresponding to the total number of 4,853 Crohn's disease (CD), 5,612 ulcerative colitis (UC) patients, and 14,239 healthy controls.

Results We confirmed that the *NKX2–3* polymorphism is associated with common susceptibility to IBD and that HLA-DRB1*0450 alleles increase susceptibility to CD but reduce risk for UC while HLA-DRB1*1502 alleles increase susceptibility to UC but reduce CD risk. Moreover, we found individual disease risk loci: *TNFSF15* and *TNFα* to CD and HLA-B*5201, and *NFKBIL1* to UC. The genetic risk of HLA was substantially high (odds ratios ranged from 1.54 to 2.69) while that of common susceptibility loci to IBD was modest (odds ratio ranged from 1.13 to 1.24).

Conclusions Results indicate that Japanese IBD susceptibility loci identified by the meta-analysis are closely associated with the HLA regions.

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Introduction

Inflammatory bowel disease (IBD) signifies a multifactorial disorder that results from an aberrant immune response to intestinal microbiota in genetically susceptible individuals [1]. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC) and the past three decades has seen an exponential increase in the incidence and prevalence of both CD and UC in Japan [2]. Familial aggregation and twin studies have suggested a genetic contribution to IBD, which appears to be less common in Japan than in Western countries [3].

In the past decade, genome-wide association studies (GWAS) focusing mainly on European descendants have advanced our understanding of the pathogenesis of IBD [4–7]. Combining previous reports with recent GWAS, indicates that a total of 163 IBD loci over genome-wide significance thresholds have been identified to date [8]. The first and best-characterized genetic susceptibility variants for CD, *nucleotide oligomerization domain 2 (NOD2)/caspase-activation recruitment domains-15 (CARD-15)*, encodes a cytoplasmic bacterial pattern-recognition receptor that senses muramyl dipeptides via its leucine-rich repeat region [9]. Another variant, *Autophagy gene autophagy-related 16-like 1 (ATG16L1)*, a gene involved in the intracellular pathogen clearance mechanism known as autophagy, was first found to be associated with the development of CD [10]. These both suggest impaired innate defense mechanisms based on normal microbial recognition and clearance determine the risk of developing CD [8]. Furthermore, proinflammatory immune responses are likely important given the association of variants in the interleukin-23 (IL-23) pathway, such as *IL12B* and *IL-23 receptor (IL23R)*, with IBD. The actions of IL-23 have largely been linked to Th17 cells, a recently discovered $CD4^+$ T cell subset thought to contribute to many immune-mediated inflammatory diseases [11]. However, there was substantial heterogeneity in the genetic structure among populations examined. Surprisingly, major disease-associated variants identified in the European sources including *NOD2/CARD15* (IBD1) [9], *solute carrier 22A4/5 (SLC22A4/5)* (IBD5) [12], and *IL23R* [13] have not been detected in the Japanese population [14–16]. This enigma may be explained by either genetic variants that are predisposed to IBD that appear to vary between different geographical and racial groups, and true culprit variants, which are common to both the disease and race, that have not been identified under the current genetic methodologies.

In Japan, there have been only four GWAS conducted on IBD genetics at present [17–20]. To our knowledge, there is no published systematic analysis of genetic variants confined to Japanese IBD. In the present study, we have conducted the first meta-analysis that assesses susceptibility genes associated with IBD in Japan. Our aim was to resolve the Japanese enigma on IBD genetics and to utilize fully past IBD genetic studies in Japan to identify Japanese population-specific or true culprit IBD susceptibility genes.

Materials and methods

Identification of relevant studies and single-nucleotide polymorphisms

The review process followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [21]. A key word search of PubMed and OvidSP, which are hosts to well-known datasets such as EMBASE, EBM Reviews, and BIOSIS Previews, and Ichushi-Web, which is a maximum-scaled Japanese medical database, was conducted using a sensitive multi-field search to identify relevant articles published through January 31, 2013. Key words utilized were: *inflammatory bowel disease* OR *IBD* AND *polymorphism* OR *gene* OR *genetics* AND *Japan* OR *Japanese*. A study's eligibility was determined through three screening steps: studies were first screened for their relevance, in the second screening appropriate single-nucleotide polymorphisms (SNPs) were chosen, and the third screening involved adjusting SNPs for the meta-analysis.

Publications meeting the eligibility criteria in the first screening step were human genetic association studies describing genotype frequencies and adopted case–control designs published in either English or Japanese. There were no publication date restrictions for these studies. Reference lists of articles retained for review and past meta-analyses were inspected for relevant publications. For multiple publications based on related data sets, the study with the greatest number of subjects was included. To maximize the number of subjects available for meta-analysis, we used 12 proxy SNPs in the 8 loci/genes obtained from previously conducted Japanese genome-wide association studies (GWAS) [17–20]. A proxy SNP whose r-squared was more than 0.9 to a particular (reference) SNP was chosen by using PLINK software (v1.07, freely available from http://pngu.mgh.harvard.edu/purcell/plink/) from the HapMap (r23) JPT-CHB genotype data [22]. Publications that were excluded from consideration consisted of review articles, studies geared toward basic science or animal research, papers analyzing unreported genotype frequencies (for example, those relying only on serological human

leukocyte antigen (HLA) typing), and phenotype-genotype association studies.

In the second screening, publications had to include the following subject matter: (1) polymorphic variants in the Japanese population; (2) SNPs satisfying the Hardy–Weinberg equilibrium (HWE); (3) statistically significant SNPs in the association studies, in which we considered statistical significance as a P value less than $P < 3.0E{-}4$ after Bonferroni's correction (0.05/168 SNPs passed by first screening); (4) more than two comparable SNPs. Concerning this final point, solitary SNPs were eliminated even if they fulfilled the first three requirements.

In the third screening, in order to minimize publication bias, SNPs that had been eliminated for not meeting the third or fourth criteria in the previous screening were reconsidered if the possibility for pairwise comparison existed. Moreover, rs2569190 within *CD14* [23] and rs763780 within *IL17F* [24], both of which were newly genotyped, were added to the analysis. Finally, to ensure the independency of the selected study subjects for the meta-analysis, any potential overlapping cohorts were discarded in consideration of Japanese districts such as Kyushu, Kanto, or Tohoku. Three reviewers extracted study information independently and disagreements were resolved through consensus.

Genotyping SNPs and unpublished data

There were 582 IBD patients (179 CD and 403 UC patients), all of unrelated Japanese descent, and 361 healthy controls were newly subjected to genotyping for the two SNPs in the present study, or rs2569190 within *CD14* and rs763780 within *IL17F*. As described in detail in the subsection 'Other loci to be explained' that appears in the RESULTS section, the pooled OR of rs2569190, from previous studies included, still indicated a marginal association with increased UC risk while rs763780 was supported by Yamazaki's unpublished sizable data. These are the reason why we should confirm these loci in the current study. The participating subjects with IBD and healthy controls were collected from Sapporo Medical University and Sapporo Kosei General Hospital in addition to subjects from our previous study [25]. The diagnosis of UC or CD in all subjects was made by expert gastroenterologists in accordance with clinical, radiological, endoscopic, and histological features according to the Lennard-Jones criteria [26]. Genomic DNA was extracted from two milliliters of peripheral blood from each participant using a NA-3000 automated nucleic acid isolation system (KURABO, Tokyo, Japan) according to the manufacturer's protocol. Extracted DNA integrity was checked by NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE). SNPs genotyping was determined by TaqMan® chemistry, by Custom TaqMan® Gene SNP Genotyping Assays, or by bidirectional PCR-direct sequence using a BigDye® Terminator Cycle Sequencing Kit with an ABI PRISM® 3100 DNA sequencer (Applied Biosystems).

Statistical analysis in the meta-analysis

The general approach to meta-analysis has been described previously [27]. The pooled frequency of the putative risk allele was estimated using the inverse variance method. Heterogeneity among studies was assessed using the Breslow–Day test [28] and I^2 statistic [29] for the allele-specific odds ratios (ORs) across studies. If no or low heterogeneity existed ($I^2 < 25$ %), the inverse variance method was used to estimate the pooled OR at a 95 % confidence interval (CI), assuming a fixed effects model. Otherwise, a random effects model was used. Since a total of 50 polymorphisms were subjected to the meta-analysis, we considered results statistically significant when the P value in the meta-analysis was less than 1.0E−3 after Bonferroni's correction (0.05/50 SNPs ultimately qualified). The test for heterogeneity between subgroup analyses was planned only with the inverse-variance method when sufficient information was reported in at least five studies in each subgroup and there was no evidence of within-group heterogeneity. Publication bias was assessed visually using a funnel plot of the standard error of the logarithm of the effect estimate against the effect estimate of each study when there were at least 10 studies included in the meta-analysis. STATA Version 10 for Windows (StataCorp LP, College Station, TX, USA) was used to conduct the meta-analysis, sequential analysis, and publication bias assessment. The HWE2 program, one of the Statistical Genetics Utility programs (provided by Jurg Ott, Ph.D., Rockefeller University and available from http://www.jurgott.org/linkage/util.htm), was used to test the departure from HWE, using a formula on page 99 in Weir [30].

Supplemental material is available online.

Ethical considerations

Written informed consent was obtained from all participants newly genotyped in this study. The study was approved by the institutional review board of Sapporo Medical University (Acceptance No. 24-17) and, therefore, was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Results

Study subjects

As detailed in Fig. 1, during the first screening, 2,633 of 2,703 (97.4 %) considered articles were rejected from subsequent analyses because they did not meet the eligibility criteria; the remaining 70 articles, whose content included 168 SNPs in 67 loci/genes, underwent the second screening. Four of the 70 articles, which included 12 SNPs in two loci/genes, were excluded due to monomorphic variants in the Japanese population. Two articles, which included examinations of 10 SNPs in four loci/genes, were excluded due to departure from HWE ($P < 3.0E{-}4$). Forty-three articles, which examined 113 SNPs in 42 loci/genes, were excluded because of their lack of significant association ($P > 3.0E{-}4$). Furthermore, five articles, which examined 25 SNPs in 14 loci/genes, were excluded because the SNPS examined were solitary, making pairwise analysis impossible. Consequently, during the second screening, 54 of the 70 (77.1 %) articles were discarded, resulting in 16 articles that included content on only eight SNPs in five loci/genes being eligible for the third screening. During the third screening, which concentrated on the formation of pairwise SNPs, 30 articles that included analysis of 43 SNPs in 18 loci/genes were reconsidered from among 43 articles focusing on 113 SNPs with no association and 5 articles examining 25 solitary SNPs. Furthermore, since rs2569190 within *CD14* and rs763780 within *IL17F* were newly genotyped and added to this analysis, 46 studies that included analysis of 51 SNPs in 23 loci/genes initially appeared to be eligible for the analysis. However, in order to avoid the potential risk of including data from overlapping cohorts, a total of 37 studies [16, 17, 25, 31–61] that included analysis of 50 SNPs in 22 loci/genes were ultimately accepted for the meta-analysis, which consisted of a total number of 4,853 CD patients, 5,612 UC patients, and 14,239 healthy controls (HCs) (Table 1).

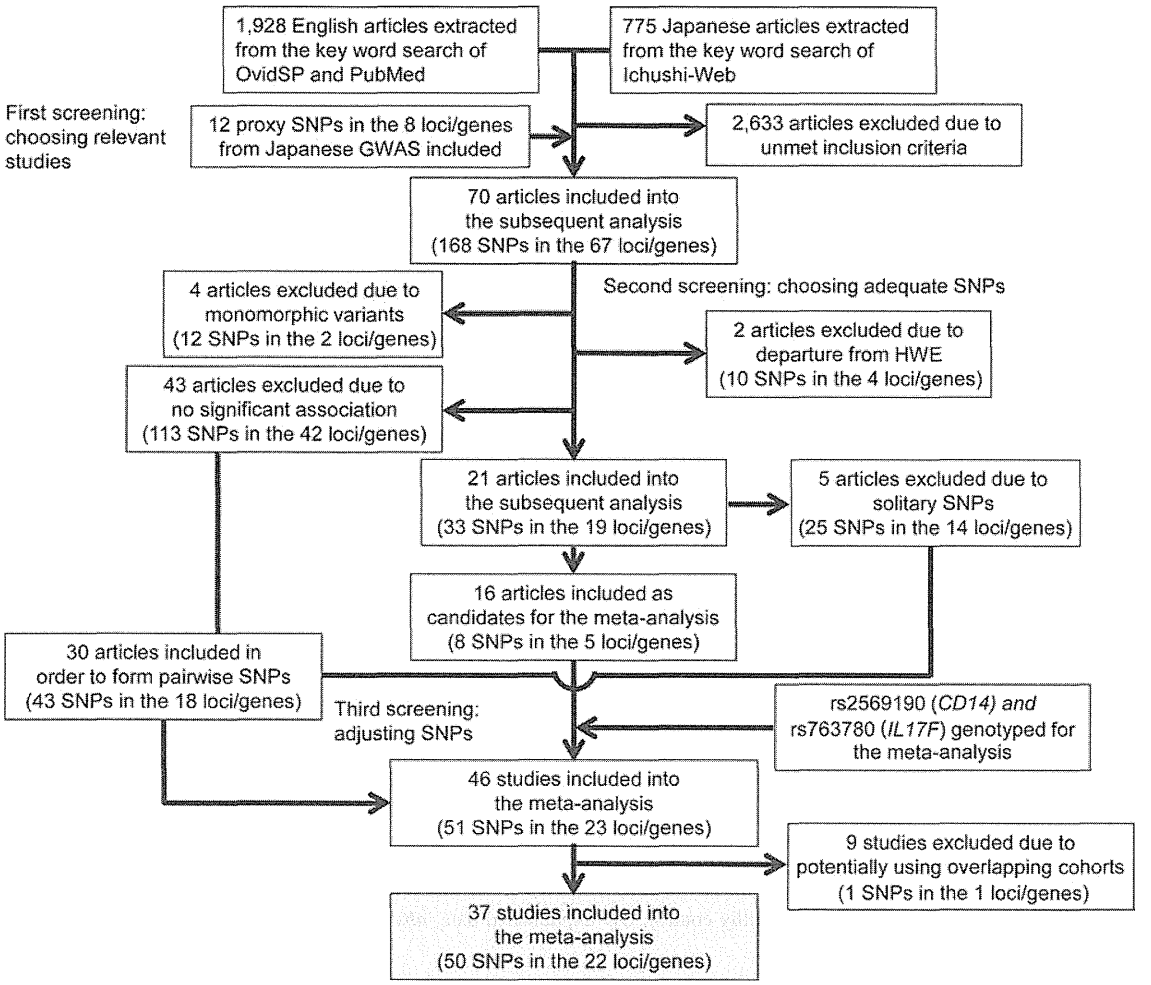

Fig. 1 Flowchart of the three screening steps of eligibility for the meta-analysis

Table 1 Studies included in the meta-analysis

No.	Author	Year	CD	UC	HC	Genes/loci	rsID	References
1	Arai	2011	344	253	243	*NKX2–3*	rs10883365, rs888208	[31]
2	Yamazaki	2009	482	0	470	*NKX2–3,10q21*	rs10883365, rs10761659	[32]
3	Tanaka	2009	165	295	393	*NKX2–3 SFTPD*	rs10883365, rs911887	[25]
4	Yamazaki	–	372	371	3366	*NKX2–3 IL-17F, TNFα PPARγ, TYK2 CTLA4 ICAM1 PTPN11 SHMT1 TLR9, CD14 NFKBIL1 IL-18*	rs888208, rs2275913 rs763780, rs2844482 rs2197423, rs280519 rs231735, rs5498 rs10850014, rs11066322 rs12952556, rs352163 rs2569188, rs1041981 rs2071593, rs1946518 rs795467	UD
5	Nakagome	2010	130	82	164	*TNFSF15 ATG16L1 IRGM, 10q21*	rs3810936, rs2241880, rs10065172 rs10761659	[33]
6	Kakuta	2006	286	263	277	*TNFSF15*	rs3810936, rs6478108, rs7848647	[34]
7	Yamazaki	2005	479	0	345	*TNFSF15*	rs3810936, rs6478108, rs7848647	[17]
8	Yamazaki	2007	481	0	437	*ATG16L1*	rs2241880	[16]
9	Prescott	2010	484	0	933	*IRGM*	rs10065172	[35]
10	Hayashi	2012	0	202	475	*IL-17A*	rs2275913	[36]
11	Arisawa	2008	0	111	248	*IL-17F*	rs763780	[37]
12	Takagawa	2005	210	205	212	*IL-18*	rs1946518	[38]
13	Tamura	2002	134	110	110	*IL-18*	rs549908	[39]
14	Sashio	2002	124	106	111	*HLA-DRB1, TNFα*	DRB1*1502, rs1800629, rs361525	[40]
15	Negoro	1999	103	76	575	*TNFα*	rs1799964, rs1800629, rs1800630, rs361525, rs1799724	[41]
16	Matsuoka	2003	76	92	99	*TNFα, CD14*	rs1799724, rs2569190	[42] (abst)
17	Aoyagi	2010	10	29	134	*PPARγ*	rs1801282	[43]
18	Wang	2008	0	118	142	*PPARγ*	rs1801282	[44]
19	Sato	2009	83	0	200	*TYK2*	rs280519	[45]
20	Machida	2005	79	108	200	*CTLA4*	rs3087243	[46]
21	Matsuzawa	2003	79	128	103	*ICAM1*	rs5498	[47]
22	Narumi	2009	83	114	200	*PTPN11*	rs2301756, rs3741983	[48]
23	Osuga	2006	0	66	173	*MDR1*	rs1045642	[49]
24	Arimura	–	0	744	1127	*SFTPD, MDR1*	rs911887, rs1045642	UD
25	Kosaka	2009	0	145	120	*SHMT1*	rs1979277	[50] (abst)
26	Shiroeda	2010	0	111	209	*MIF*	rs755622	[51]
27	Nohara	2004	0	221	438	*MIF*	rs755622	[52]
28	Fuse	2010	0	48	47	*TLR9*	rs187084, rs352139, rs352140	[53]
29	Wang	2007	0	97	135	*CD14*	rs2569190	[54]
30	Obana	2002	82	101	123	*CD14*	rs2569190	[55]
31	Oomori	2009	0	250	303	*NFKBIL1 (IKBL)*	rs3219184, rs2071592	[56]
32	Okada	2011	372	372	905	HLA-B HLA-DRB1	B*4002, *5201, DRB1*1502, *0405 *1501	[19]
33	Aizawa	2009	0	318	319	HLA-B	*4002, *5201	[57]
34	Kinouchi	2003	195	0	185	HLA-B	*4002, *5201	[58]
35	Seki	2001	0	64	236	HLA-B	*5201	[59]
36	Futami	1995	0	59	150	HLA-DRB1	*1502, *0405, *1501	[60]
37	Matsumura	2008	0	353	332	HLA-DRB1	*1502	[61]
	Total		4,853	5,612	14,239	22	50	

For multiple SNP genotyping based on related data sets, the study population with the greatest number of subjects was represented

CD Crohn's disease, *UC* ulcerative colitis, *HC* healthy control, *UD* unpublished data, *abst* abstract

Japanese IBD susceptibility candidate loci

The rs10883365 in *NK2 transcription factor-related, locus 3* (*NKX2–3*) was identified as a candidate IBD common susceptibility loci; the estimated pooled odds ratio for CD susceptibility was 1.13 and the 95 % confidence interval (95 % CI) was 1.05–1.22, which was considered as statistically significant ($P = 9.5E{-}4$). For UC, the odds ratio was 1.24 (95 % CI 1.13–1.36), which was statistically significant ($P = 9.0E{-}6$, Fig. 2). However, rs888208 in *NKX2–3* was not strictly identified as an IBD susceptibility candidate locus due to its marginal significance ($P = 0.005$ for CD and $P = 0.002$ for UC). The rs3810936 within the *tumor necrosis factor (ligand) superfamily, member 15* (*TNFSF15*) was identified as one of the CD susceptibility candidate loci; the estimated pooled odds ratio for CD susceptibility was 1.23 and the 95 % confidence interval (95 % CI) was 1.17–1.30, which was statistically significant ($P = 6.5E{-}14$). For UC susceptibility, it was 1.10 (1.02–1.19) of an overall estimated pooled odds ratio, which did not reach statistical significance ($P = 0.008$) (Fig. 2).

Japanese CD susceptibility candidate loci

Compared to the controls, HLA-DRB1*0405 allele significantly increased susceptibility to CD (OR 1.54, 95 % CI 1.34–1.75, $P = 2.7E{-}10$), but reduced risk for UC (OR 0.74, 95 % CI 0.60–0.91, $P = 0.005$) (Fig. 3). Other CD-specific candidate loci were rs1799964 (OR 1.31, 95 % CI 1.05–1.63, $P = 3.0E{-}4$) and rs1800630 (OR 1.34, 95 % CI 1.07–1.67, $P = 2.0E{-}4$), both of which were located within the *tumor necrosis factor α* (*TNFα*) locus (Fig. 3).

Japanese UC susceptibility candidate loci

Compared with the controls, HLA-B*5201 and HLA-DRB1*1502 alleles significantly increased susceptibility to UC (OR 2.69, 95 % CI 2.24–3.23, $P = 1.9E{-}43$ and OR 2.17, 95 % CI 1.63–2.90, $P = 1.3E{-}37$, respectively), but reduced risk for CD (OR 0.64, 95 % CI 0.45–0.90 $P = 0.010$ and OR 0.49, 95 % CI 0.36–0.66, $P = 1.1E{-}12$). Other UC-specific candidate loci were rs2071592 (OR 1.14, 95 % CI 1.09–1.19, $P = 4.5E{-}9$) and rs3219184 (OR 1.93, 95 % CI 1.75–2.13, $P = 1.3E{-}38$), both of which were located within the *nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1* (*NFKBIL1*) locus (Fig. 4).

Other loci to be explained

The pooled OR from all included studies still indicated a marginal association between *CD14* (C-260-T

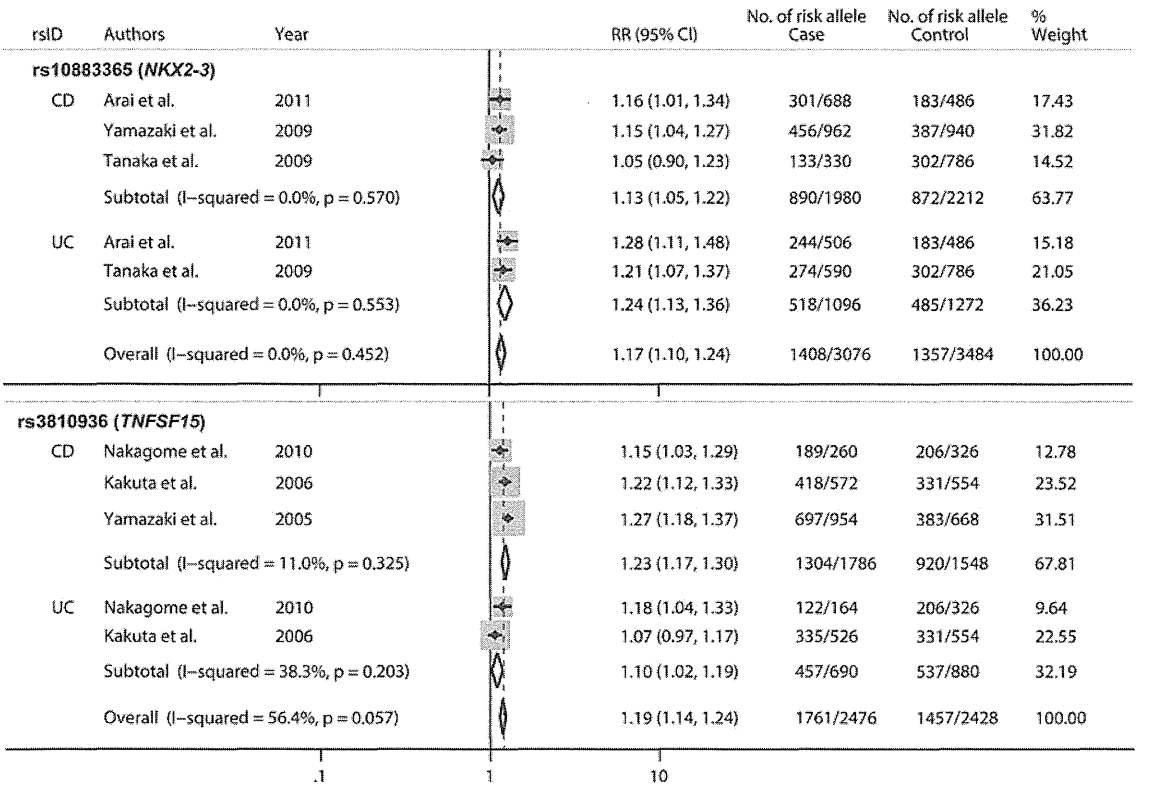

Fig. 2 IBD common candidate loci. *NKX2–3*, *NK2 transcription factor-related, locus 3*, *TNFSF15*, *tumor necrosis factor* (*ligand*) *superfamily, member 15*

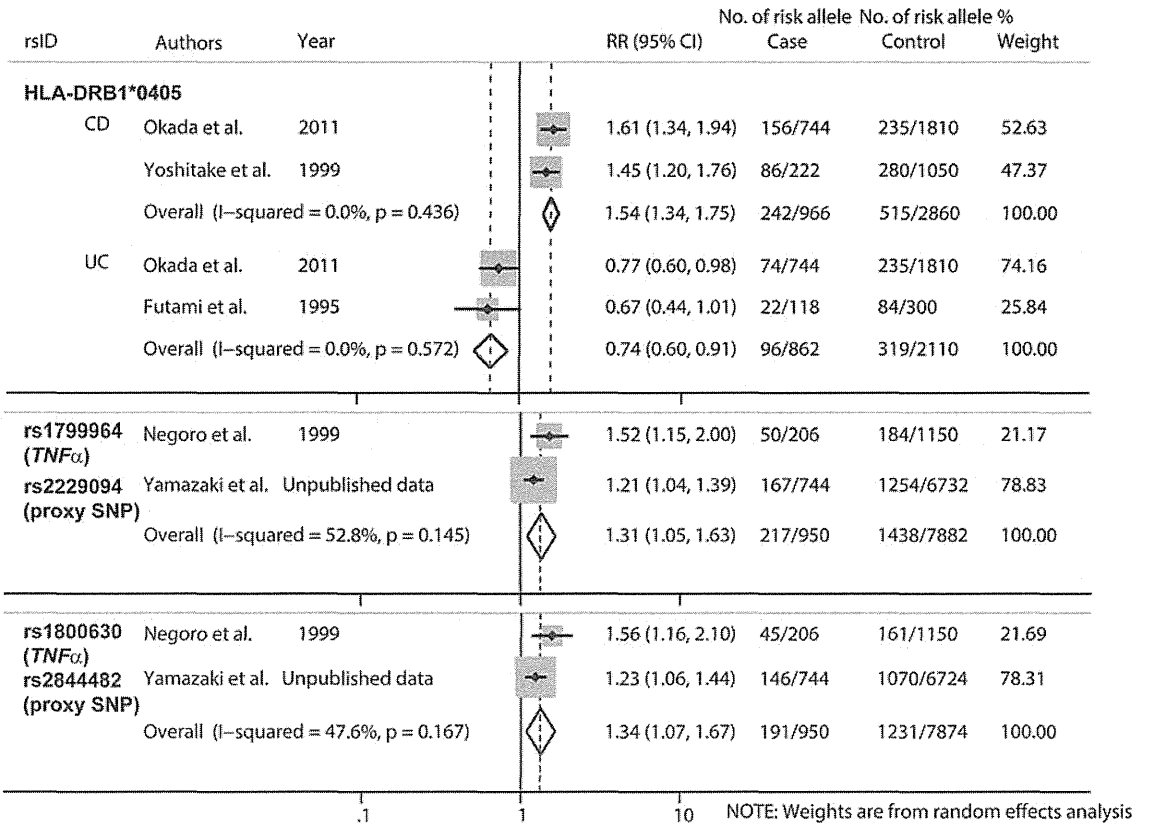

Fig. 3 CD candidate loci. *TNFα, tumor necrosis factor α*

polymorphism, rs2569190) polymorphism, and increased UC risk in allelic comparison (OR 1.14, 95 % CI 1.01–1.29, $P = 0.005$, Fig. 5), although there was strong evidence of between-study heterogeneity ($I^2 = 76.5$ %, $P = 0.002$). It should be noted that similar to the result of the current study, recent studies including Yamazaki's unpublished data have failed to show any statistical significance. These findings strongly suggested that this polymorphism might be a false positive variant in Japanese susceptibility to UC although in a previous meta-analysis, Wang et al. [23] reported that the polymorphism showed potential to be a candidate marker regarding susceptibility to UC, particularly among Asians. Evaluations of *IL17F* (rs763780), *MDR1* (rs1045642), and *SFTPD* (rs911887) produced conflicting results although there was strong evidence of between-study heterogeneity in these studies (I^2 more than 80 %, $P = 0.001$–0.023). While the *IL17F* polymorphism among the above three genes was supported by Yamazaki's unpublished data, her sample size of 371 UC patients and 3,365 healthy controls was so significant that the weight accounted for 63.8 % of all the studies; as such, the replication study we conducted here rejected Yamazaki's association. Moreover, the association between *MDR1* polymorphism and UC risk was also unconfirmed in our study. Unfortunately, although we previously reported *SFTPD* as a possible UC susceptibility variant candidate [25], this study clearly shows that it was a false-positive result from a candidate approach even though the study was carefully conducted with the latest knowledge on linkage disequilibrium (LD) of the interest loci (Fig. 5).

Detailed results including individual combined *P* values obtained by the meta-analysis are displayed in Supplementary Table 1.

Discussion

A total of 2,703 previous studies related to Japanese IBD genetics were identified by our review process. Only 37 of these studies (1.4 %), which included analysis on 50 SNPs within 22 loci, met all the criteria to be included in the meta-analysis (Table 1). The results obtained from the current meta-analysis (Fig. 6) can be summarized as follows. First, *NKX2–3* (rs10883365) and potentially *TNFSF15* (rs3810936) are shown to be common susceptibility genes for Japanese IBD, whose individual effect size is quite modest, i.e., odds ratio of less than 1.2 (1.09–1.19).

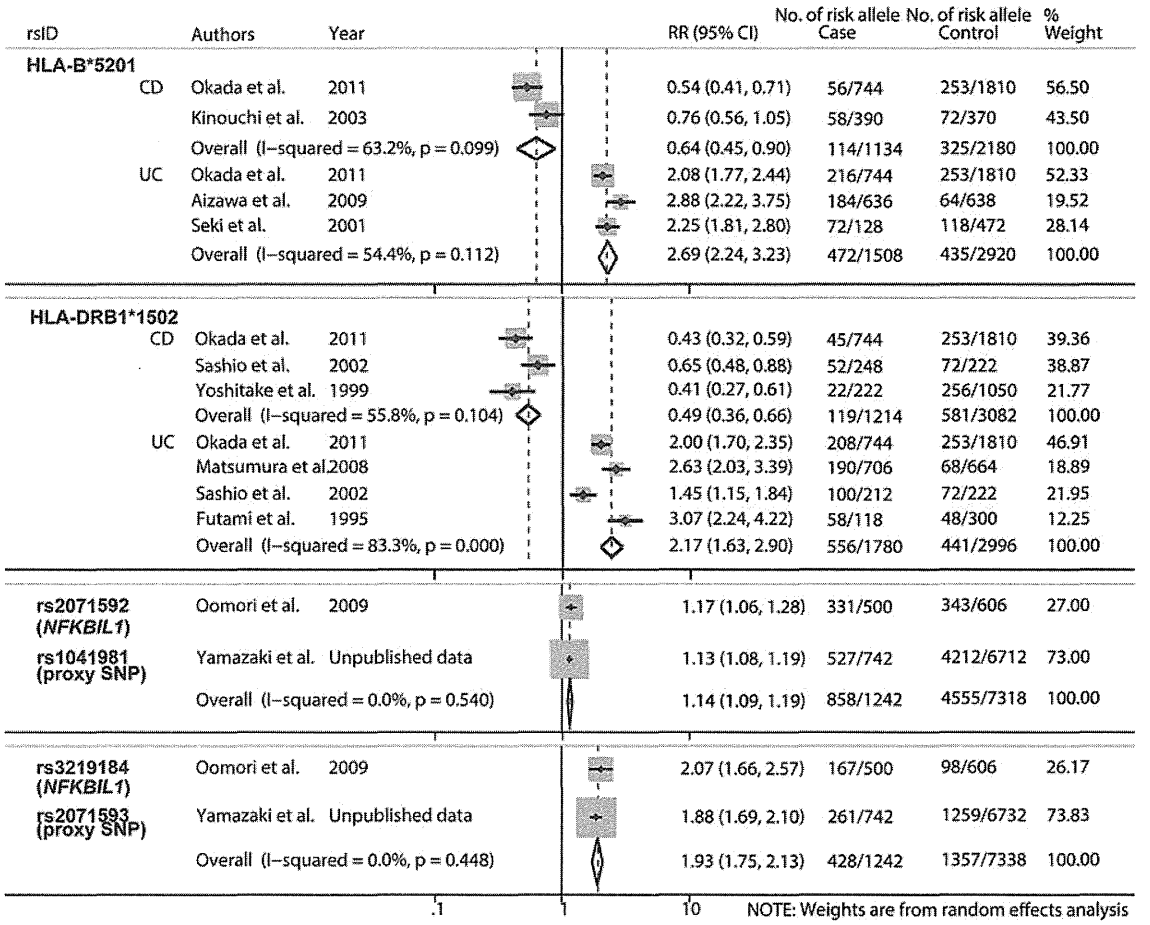

Fig. 4 UC candidate loci. *NFKBIL1*, *nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1*

Since these genes were previously recognized as Caucasian IBD susceptibility genes [5, 62], they are accepted as being rare candidates of common IBD susceptibility genes extending beyond racial differences. Second, several HLA alleles have opposing effects on disease risk with a relatively large effect size implying potential use as diagnostic genetic markers for IBD; the HLA-DRB1*0405 allele increases susceptibility to CD but reduces risk for UC. In contrast, HLA-B*5201 and DRB1*1502 alleles increase susceptibility to UC but reduce risk for CD. Third, individual CD- or UC-specific susceptibility candidate variants are identified: *TNFα* (rs1799964 and rs1800630) to CD and *NFKBIL1* (rs2071592 and rs3219184) to UC. Finally, *SFTPD* (rs911887), which we previously reported as a possible Japanese UC susceptibility gene [25], could not be replicated by the other replication set of the Japanese population.

Combining previous studies with our analysis, both *TNFSF15* and *NKX2–3* can be considered IBD common risk loci for the Japanese population. *TNFSF15* is the first CD susceptibility gene discovered in Japan that is also confirmed in Caucasian populations [17, 62]. *TNFSF15* encodes a tumor necrosis factor-like cytokine, *TL1A*, which drives mucosal inflammation by signaling via the transcription factor NFκB and can enhance the mucosal function of Th1 and Th17 cells [63]. In contrast, *NKX2–3* is a member of the NKX homeodomain transcription factor families, which is a gene associated with the development of a correct environment for B-cell- and T-cell-dependent immune responses [64–68]. Unfortunately, the individual effect size of these polymorphisms is quite modest, and the true culprit loci with larger effect size across the different backgrounds has yet to be identified.

Recently, Okada et al. [19] has reported that the long-range HLA-Cw*1202-B*5201-DRB1*1502 haplotype throughout the MHC telomeric class I and III regions, and the centromeric II region increases susceptibility to UC but reduces risk for CD. According to Okada, DRB1*0405 shows a significant association between UC and CD ($P = 2.4E{-}8$), with a susceptible effect on CD ($P = 3.8E{-}7$) and no significant association with UC ($P = 0.072$). These results are compatible with previously reported

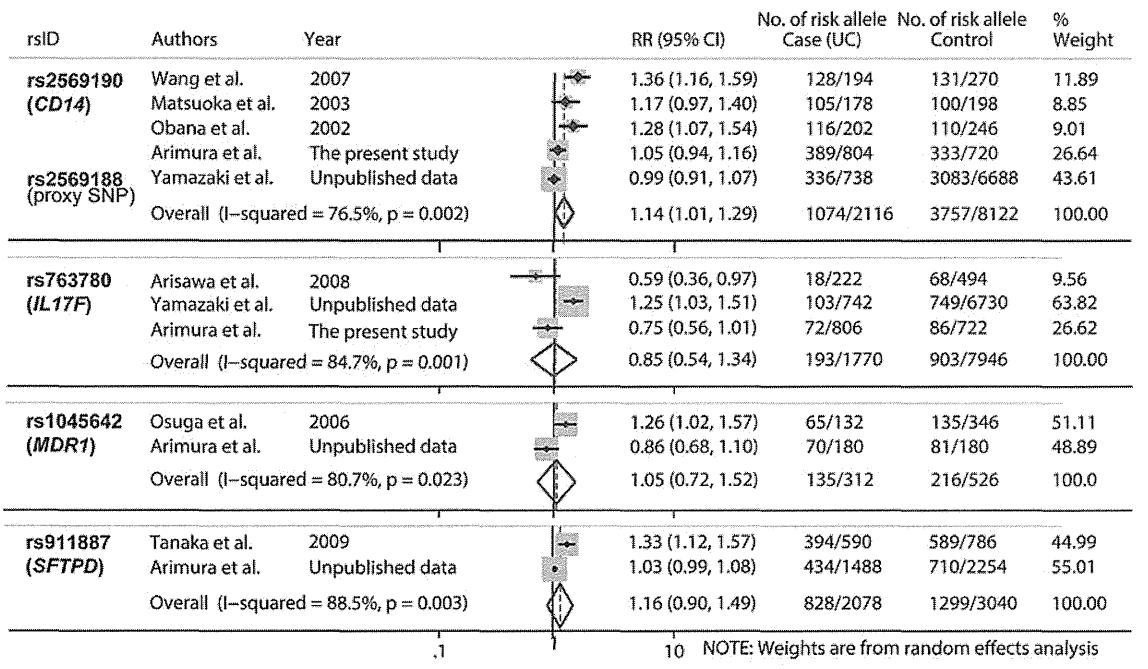

Fig. 5 Other potential UC candidate loci. *MDR1*, *multidrug resistance protein 1*; *SFTPD*, *surfactant, pulmonary-associated protein D*

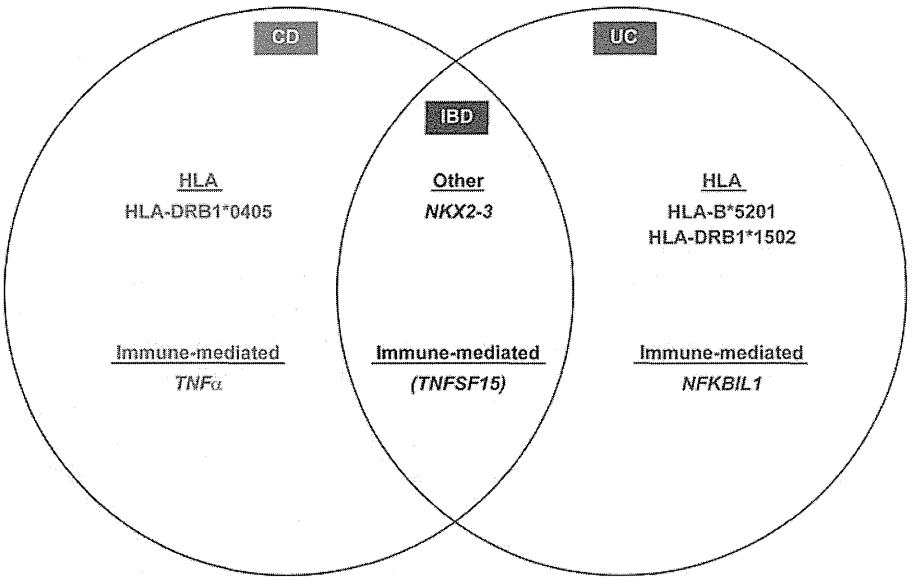

Fig. 6 Japanese IBD susceptibility loci. The loci (depicted by lead gene name) attaining statistical significance in the meta-analysis ($P < 1.0E-3$) are shown for Crohn's disease (*red*), ulcerative colitis (*blue*) and IBD (*black*). rs3810936 within the *tumor necrosis factor (ligand) superfamily, member 15* (*TNFSF15*) was identified as one of the CD susceptibility candidate loci while it did not reach statistical significance ($P = 0.008$) for UC susceptibility in the meta-analysis. However, since *TNFSF15* was previously recognized as one of the Caucasian IBD susceptibility genes [5, 62], it is accepted as being a rare candidate of common IBD susceptibility genes extending beyond racial differences and is noted in brackets here

associations of B*5201 and DRB1*1502 with UC [59, 69, 70] or DR4 alleles with CD [69, 70]. In our analysis, B*5201 and DRB1*1502 alleles also exhibit behavior suggesting that they should form the haplotype. Moreover, along the lines of Okada's report, in our analysis ($P = 2.7E-10$) DRB1*0405 significantly increased risk for CD, in which the pooled odds

ratio (95 % CI) is 1.54 (1.34–1.75). In contrast, UC susceptibility was not increased.

Except for HLA alleles and IBD common loci such as *TNFSF15* and *NKX2–3*, all of which are statistically significant in the meta-analysis ($P < 1E-3$), rs1799964 and rs1800630 in *TNFα* are associated with CD susceptibility while rs2071592 and rs3219184 in *NFKBIL1* are associated with UC susceptibility. Moreover, the *P* value of *NFKBIL1* association is less than the 5E−8 that is considered the standard for genome-wide significance. Since both *TNFα* and *NFKBIL1* genes are located in the class III region near the border of the class I region, these assumed associations should be seriously affected by the long-range HLA-Cw*1202-B*5201-DRB1*1502 haplotype reported by Okada et al. These results indicate that most Japanese IBD susceptibility loci identified by the meta-analysis are closely associated with HLA regions.

There are, of course, drawbacks in our meta-analysis. IBD susceptibility loci recently identified by Japanese GWAS were ultimately excluded from the meta-analysis because replication studies on these recent loci have not yet been reported in Japan. For example, there have been no studies on UC susceptibility loci such as the immunoglobulin receptor gene *FCGR2A* (rs1801274), a locus on chromosome 13q12 (rs17085007), or the glycoprotein gene *SLC26A3* (rs2108225) [18]. Moreover, two CD susceptibility loci on chromosome 4p14 (rs1487630) and in the *SLC25A15-ELF1-WBP4* region on 13q14 (rs7329174 in *ELF1*) [20] have only been reported on very recently. It is important that these loci be systematically analyzed in future Japanese nation-wide replication studies.

Major key tasks for the current IBD genetics are GWAS and fine mapping both describing Asian populations. It is believed that just as NOD2 appears to be a European ancestral specific gene, ancestral specific loci may exist for Asian populations. However, there are only five GWAS, including the four from Japan [17–20] and the most recent one from Korea [71], and one meta-analysis [72] concerning Asian IBD genetics at present. Accordingly, we summarized in detail the comparison between the Asian and current Japanese meta-analysis in Table 2 [72]. This comparison clarified that although genes identified as significant were similar between the two meta-analyses, there were substantial differences from exact susceptible loci in each gene. The Japanese results were more conservative than those in the Asian meta-analysis. Half (5 loci) of the total of 10 loci compared was insignificant in Japanese IBD: rs6478108 and rs7848647 in *TNFSF15*, *TNFα* and $(AT)_n$ in 3′-UTR and rs3087243 in *CTLA4*. This for the first time suggests that we should not always analyze Asian and Japanese genetic data indiscriminately to deal with such a delicate issue when exploring the Japanese population specific culprit susceptibility genes.

Furthermore, we summarized in detail the comparison between the Caucasian and current Japanese meta-analysis in Table 3 [7, 73–75]. The comparison revealed that *NFKBIL1* and *CD14* were not identified as UC susceptibility genes in Caucasian populations, and there were substantial differences in the culprit HLA regions as previously reported [75]. For example, HLA-DRB1*1502 and HLA-DRB1*0103 have shown consistent association with UC [60, 76]. HLA-DRB1*0103 represents the most

Table 2 Summary of meta-analyses between Asian and Japanese IBD genetics

Genes/loci	rsID	IBD	No. of studies	Odds ratio (95 % CI)	*P* value
ATG16L1	rs2241880	CD	3	0.97 (0.84–1.13)	0.69 [72]
	rs2241880	**CD**	**2**	**0.94 (0.82–1.08)**	**0.38**
IL-23R	rs1004819	CD	1 (Yang 2009)	1.82 (1.16–2.85)	0.73 [72]
	rs1004819	**CD**	**1** [16]	**NA**	**0.59**
TNFSF15	rs3810936	CD	2	2.39 (1.85–3.10)	<1.0E−5 [72]
	rs3810936	**CD**	**3**	**1.23 (1.17–1.30)**	**6.5E−14**
	rs6478108	CD	2	2.84 (1.97–4.09)	<1.0E−5 [72]
	rs6478108	**CD**	**2**	**0.97 (0.55–1.71)**	**0.92**
	rs7848647	CD	2	2.72 (1.73–4.30)	<1.0E−4 [72]
	rs7848647	**CD**	**2**	**0.819 (0.48–1.79)**	**0.82**
TNFα	rs1800629	UC	6	1.82 (1.15–2.94)	0.01 [72]
	rs1800629	**UC**	**2**	**0.716 (0.06–63.75)**	**0.716**
CTLA4	N.A.	UC	3	2.75 (1.22–6.22)	0.01 [72]
	rs3087243	**UC**	**2**	**1.04 (0.93–1.15)**	**0.53**
HLA	DR2 and DRB1*1502	UC	4	2.41 (1.89–3.07)	<1.0E−5 [72]
	DRB1*1502	**UC**	**4**	**2.17 (1.63–2.90)**	**1.3E−37**

Data of bold rows are observed in the current study

Table 3 Summary of meta-analyses between Caucasian and Japanese IBD genetics

Genes/loci	rsID	IBD	Odds ratio (95 % CI)	*P* value
NKX2–3	rs10883365	CD	1.21 (1.04–1.40)	4.0E−5 [73]
		CD	**1.13 (1.05–1.22)**	**9.5E−4**
		UC	1.31 (1.10–1.56)	2.4E−7 [73]
		UC	**1.24 (1.13–1.36)**	**9.0E−6**
TNFSF15	rs3810936	CD	1.21 (1.15–1.27)	1.0E−15 [7]
		CD	**1.23 (1.17–1.30)**	**6.5E−14**
		UC	1.12 (1.07–1.17)	1.3E−7 [74]
		UC	**1.10 (1.02–1.19)**	**0.008**
TNFα	rs1799964	CD	1.19 (1.13–1.25)	4.0E−11 [7]
		CD	**1.31 (1.06–1.63)**	**3.0E−4**
	rs1800630	CD	1.53 (1.22–1.92)	1.4E−5 [75]
		CD	**1.34 (1.08–1.67)**	**2.0E−4**
NFKBIL1	rs2071592	UC	NA	NA
		UC	**1.14 (1.09–1.19)**	**4.5E−9**
	rs3219184	UC	NA	NA
		UC	**1.93 (1.75–2.13)**	**1.3E−38**
HLA	DRB1*0405	CD	1.97 (1.62–2.40)	* [75]
		CD	**1.56 (1.34–1.76)**	**2.7E−10**
		UC	0.49 (0.31–0.76)	NA [75]
		UC	**0.74 (0.60–0.91)**	**0.005**
	B*5201	CD	NA	NA
		CD	**0.64 (0.45–0.90)**	**0.010**
		UC	3.35 (2.23–5.01)	* [75]
		UC	**2.33 (1.94–2.79)**	**1.9E−43**
	DRB1*1502	CD	0.61 (0.50–0.78)	NA [75]
		CD	**0.49 (0.36–0.66)**	**1.1E−12**
		UC	3.24 (2.54–4.13)	* [75]
		UC	**2.17 (1.63–2.90)**	**1.3E−37**
CD14	rs2569190	UC	NA	NA
		UC	**1.14 (1.01–1.29)**	**0.005**
IL17F	rs763780	UC	1.02 (0.97–1.07)	0.445 [74]
		UC	**0.85 (0.54–1.34)**	**0.488**
MDR1	rs1045642	UC	1.03 (0.98–1.07)	0.172 [74]
		UC	**1.05 (0.72–1.52)**	**0.802**
SFTPD	rs911887	UC	1.02 (0.98–1.07)	0.264 [74]
		UC	**1.16 (0.90–1.49)**	**0.260**

Data of bold rows are observed in the current study. It notes that unavailable data (NA) in a meta-analysis strongly suggest they are not susceptibility genes and discarded from the analysis

NA not available

* Significant but uninformative *P* value

reproducible association observed to date in Caucasian UC [77], but not in Japanese UC. On the contrary, HLADRB1*1502 has shown association to UC in the Japanese population, where it is highly prevalent (20–25 %) [57–61], but also in European populations, where it is rare (<1 %) [78]. These observations help shed light on ancestral specific loci existing for Japanese as well as Asian populations.

The Japanese enigma on IBD genetics finally consolidates in the HLA region, which is highly polymorphic, and held in extensive linkage disequilibrium and the area in which numerous immune-related genes in high density are located. Therefore, previous IBD genetic studies in Japan are unfortunately now of little use when it comes to identifying Japanese population-specific or true culprit IBD susceptibility genes. We should approach the topic of formidable HLA regions. Indeed, advancing microbiome technology has recently unveiled the interaction of HLA genotypes with microbiota as a representative environmental factor. For example, T cell responsiveness on the specific microbiota depends on HLA genotypes [79]. Furthermore we should focus simultaneously on rare SNP variants based on common disease rare variant hypotheses and single nucleotide variants (SNV) other than SNPs such as substitutions, deletion insertion polymorphisms (DIPs), large insertion deletions (INDELs), and copy number variant (CNV). Since only 20–30 % of the genetic contribution on the IBD development could be explained by the current GWAS, missing heritability is still an open question in IBD genetics. Epigenetics has attracted considerable attention because of its potential for elucidation in the gene–environmental interaction [80]. Indeed, when compared to those of healthy volunteers, many DNAs of immune-related genes in the Th17 pathway have been proved to be distinctly methylated in IBD patients as well as in rheumatoid arthritis patients [81, 82].

In summary, functional studies on epistasis and gene–environmental interactions should be elucidated by cracking the genetic and epigenetic code of HLA regions. We believe such coordinated efforts could resolve not only the Japanese question of IBD susceptibility but could also explain the missing heritability in worldwide IBD genetics.

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Serum microRNA levels in patients with Crohn's disease during induction therapy by infliximab

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Abstract

Background and Aim: microRNAs (miRNAs) have been suggested to be candidates for biomarkers in various diseases including Crohn's disease (CD). To identify possible biomarkers predictive of the therapeutic effect of infliximab in CD, we investigated serum miRNA levels during the induction therapy by the medication.

Methods: Nineteen CD patients who were applied to the induction therapy by infliximab were enrolled. Serum samples for miRNA analyses were obtained at weeks 0 and 6, and the therapeutic efficacy by infliximab was assessed according to the Crohn's disease activity index value at week 14. Exploratory miRNA profiling by low-density array was initially performed in three patients. The levels of candidate miRNA were subsequently determined by real-time polymerase chain reaction (PCR) assays in the remaining 16 patients. The miRNA levels during the induction therapy were compared between the two groups classified by the clinical response to infliximab at week 14.

Results: Low-density array analysis identified 14 miRNAs that showed twofold or more altered expression during the induction therapy by infliximab. Subsequent analysis by real-time PCR demonstrated significantly increased levels of five miRNAs (let-7d, let-7e, miR-28-5p, miR-221, and miR-224) at week 6 when compared with those at week 0 ($P < 0.05$ each). In addition, miRNA levels of let-7d and let-7e were significantly increased in the group of patients who achieved clinical remission by infliximab ($P = 0.001$ and $P = 0.002$, respectively).

Conclusion: let-7d and let-7e might be possible therapeutic biomarkers in patients with CD, who are treated by infliximab.

Introduction

Crohn's disease (CD) is a chronic inflammatory disease characterized by transmural inflammation involving any part of the gastrointestinal tract. If not promptly or adequately treated, the disease may cause various intestinal complications, such as stricture, perforation, fistula, and abscess, which may require surgical interventions. However, the advent of infliximab (IFX) has dramatically improved medical management in patients with CD, and the biological therapy has now become the mainstay for the induction and the maintenance therapy for the disease.[1] However, IFX has been reported to be ineffective for the induction of remission in one third of the patients.[2] Furthermore, loss of response to IFX occurs in up to 50% of CD patients during the maintenance therapy.[3] Hence, factors associated with less therapeutic effect of IFX have been rigorously investigated from the clinical,[4,5] immunological,[6,7] and genetic viewpoints.[8,9]

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that lead to translational repression and degradation of target genes,[10] and they have been considered to play crucial roles in the pathophysiology of various diseases through multiple biological processes, such as cell differentiation, growth, and apoptosis.[11] Since miRNAs have also been detected as a stable form in human circulating blood,[12–15] circulating miRNAs have been noted as possible noninvasive biomarkers in various diseases such as cancer,[16,17] cardiovascular disease,[18] and autoimmune diseases.[19,20] As for inflammatory bowel diseases (IBDs), distinct miRNAs expression has been demonstrated in intestinal tissues of ulcerative colitis (UC) and CD patients,[21–24] with an emphasis of the association between miR-192 and the colonic cell derived chemokine MIP-2α (CXCL2).[21] Although Zahm *et al.* previously investigated the diagnostic potential of serum miRNAs in pediatric CD patients,[25] the pathophysiological role of serum miRNAs with a reference to a specific treatment has not been analyzed in adult CD patients.

In the present study, we investigated serum miRNA levels during the induction therapy by IFX in CD patients in order to identify possible biomarkers, which are predictive of the short-term therapeutic effect of IFX.

Methods

Patients and study protocol. This was a prospective single-center study conducted at our institution from February 2009 to March 2012. CD patients who were applied to induction therapy by IFX were the subjects of the present investigation. At the enrollment, patients who had a prior history of IFX and/or other biologics administration were excluded. Patients receiving concurrent treatment of immunosuppressive drugs or corticosteroid were included in the present study when the dosage had been stable for at least 8 weeks before IFX administration. The diagnosis of CD was based on clinical, radiological, endoscopic, and pathological findings using established criteria.[26]

The induction therapy was composed of the administration of IFX (5 mg/kg) at weeks 0, 2, and 6. All of the patients received a single dose of methylprednisolone (125 mg intravenously), acetaminophen (400 mg orally), and diphenhydramine (40 mg orally) before the administration of IFX so that the risk of an immediate infusion reaction could be minimized. Patients were followed for 14 weeks after the first administration of IFX, and the therapeutic efficacy of the induction therapy was assessed at the end of the follow up. Patients who could achieve a Crohn's disease activity index (CDAI) value less than 150 at week 14 were classified into the remission group (R-group). Patients who had CDAI value of 150 or more at week 14, or who required a rescue therapy before week 14, were classified into no-remission group (N-group). Blood samples for routine laboratory tests were obtained at each clinical visit, and those for miRNAs analyses were obtained at weeks 0 and 6.

The study protocol was approved by the ethical committee at Kyushu University Hospital, and the study was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all the enrolled subjects.

Preparation of serum samples and total RNA extraction. After stabilizing at room temperature for at least 30 min, blood samples were centrifuged at 1600× g for 15 min at 4°C. Isolated sera were aliquoted into microcentrifuge tubes and stored at −80°C until further procedure.

Two hundred fifty microliter of each serum was thawed on ice and mixed thoroughly with 750 μL of Isogen-LS (Nippongene, Toyama, Japan). After incubation at room temperature for 5 min to deactivate the RNase function completely, 250 amol (total volume of 5 μL) of synthetic *Caenorhabditis elegans* miRNA cel-miR-39 (synthesized by Hokkaido System Science, Sapporo, Japan) were added to each denatured sample as the spiked-in control.[13] Two hundred microliter of chloroform were added and tubes were shaken vigorously, then samples were centrifuged at 12 000× g for 15 min at 4°C. Thereafter, about 500 μL of aqueous phase were carefully transferred into new tubes and mixed with 1.5 volumes of 100% ethanol. RNA purification was performed using miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and the final elution volume was 50 μL.

Serum miRNAs profiling by low-density array platform. Three microliter of total RNA was mixed with Taqman microRNA Reverse Transcription Kit and Megaplex RT primers Human pool A v2.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendation. For reverse transcription, mixture was set on GeneAmp PCR system 9700 (Applied Biosystems) as follows: 40 cycles of 16°C for 2 min, 42°C for 1 min, and 50°C for 1 s, then incubation at 85°C for 5 min. Generated complementary DNA was pre-amplified by Megaplex PreAmp primer, Human Pool A, and PreAmp Master Mix (Applied Biosystems) according to the manufacturer's instructions. The pre-amplification reaction was performed by the incubation of samples, consisted of 25-μL mixture, at 95°C for 10 min, 55°C for 2 min, 72°C for 2 min, followed by 12 cycles of 95°C for 15 s and 60°C for 4 min. After the pre-amplification cycle, samples were heated at 99.9°C for 10 min to deactivate the enzyme function, then held at 4°C. Pre-amplified products were diluted by 0.1 × Tris-EDTA (pH 8.0) to a final volume of 100 μL.

The expression profile of miRNAs was determined by Taqman Array Human MicroRNA A Card v2.0 (Applied Biosystems), which is capable of detecting 377 mature human miRNAs based on real-time polymerase chain reaction (PCR) technique. Reaction solution consisted of 450 μL of TaqMan Universal Master Mix II No UNG (Applied Biosystems), 9 μL of diluted pre-amplified product and 441 μL of nuclease-free water. Reaction was carried out on 7900HT Fast Real-Time PCR System (Applied Biosystems) with manufacturer's recommended program.

Validation by real-time PCR. Taqman microRNA Reverse Transcription Kit and Taqman miRNA Assays (Applied Biosystems) were used for individual miRNAs tests. Reverse transcription and real-time PCR were performed in the scaled-down condition. In brief, 2.5 μL of total RNAs were mixed with 0.75 μL of 10 × reverse transcription buffer, 0.095 μL of RNase inhibitor, 0.075 μL of dNTPs with dTTP, 0.5 μL of Multiscribe reverse transcriptase, 1.5 μL of miRNA-specific stem-loop RT primer, and 2.08 μL of nuclease-free water. The mixture was incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. After preserving at −30°C for several days, 0.67 μL of generated complementary DNA solution was mixed with 5.0 μL Taqman Universal Master Mix II No UNG, 0.5 μL of primer and probe set, and 3.83 μL of nuclease-free water. Real-time PCR was performed on 7500 Real-Time PCR System (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each reaction was performed in triplicate. The cycle threshold (C_T) values were calculated with SDS 2.0.5 software and each miRNA level was normalized to the level of cel-miR-39.

Statistical analysis. Clinical characteristics were compared using Fisher's exact test, chi-square test, or Mann–Whitney *U*-test where appropriate. Paired samples within the same subjects were compared by Wilcoxon signed-rank test, while unpaired samples were compared by Mann–Whitney *U*-test. Spearman's rank correlation coefficient was used for the assessment of correlation between the continuous variables. A P value less than 0.05 was regarded as statistically significant for each test.

Results

Clinical characteristics of CD patients. We recruited 21 Japanese CD patients for the present study. However, two patients were excluded, one for acute pancreatitis after the second infusion of IFX, and the other for severe hemolysis of the collected sample. Thus, the remaining 19 patients were the subjects of the present analyses. Among these subjects, we chose three newly diagnosed CD patients for exploratory array test, who responded to the induction therapy by IFX alone. In the patients, the mean CDAI value decreased from 186.5 ± 10.8 at baseline to 61.1 ± 14.8 at week 6. The mean C-reactive protein (CRP) levels decreased from 0.56 ± 0.24 at baseline to 0.11 ± 0.03 at week 6. The other 16 patients were allocated to the validation experiment by real-time PCR.

Table 1 summarizes baseline characteristics of the validation cohort. Of the 16 patients, the induction therapy by IFX resulted in clinical remission in 11 patients (R-group), while the remaining five patients failed to achieve clinical remission by IFX (N-group). Although there were no significant difference of CDAI values and serum CRP levels at baseline between two groups, the time after initial diagnosis of CD was significantly longer in N-group than in R-group ($P = 0.005$). In addition, patients having history of intestinal resection were more frequent in N-group than in R-group ($P = 0.013$). There was a trend toward a higher frequency of antibiotics therapy in N-group; however, no significant difference was observed between the two groups concerning other medical treatment.

Table 2 shows CDAI values and serum CRP levels of R-group and N-group at each time of assessment. Although serum CRP levels at weeks 2 and 6 temporarily decreased in N-group ($0.05 < P < 0.1$), the value regained at week 14. CDAI values in N-group were not different at each assessment period.

Screening for candidate miRNAs. Exploratory miRNAs profiling by low-density array platform was initially performed using six samples obtained from three subjects (at weeks 0 and 6). When C_T values < 33 were regarded as positive, the profiled data identified 126 miRNAs at least in four samples. We

Table 1 Patients' characteristics of the validation cohort

	R-group (n = 11)	N-group (n = 5)	P value
Gender, male/female	11/0	3/2	0.083
Age (years)	24 (17–38)	29 (23–69)	0.053
Time after initial diagnosis of CD (years)	0.5 (0.2–8)	12 (4–39)	0.005
Involved site			0.14
Ileum	7	1	
Ileum and colon	3	4	
Colon	1	0	
History of intestinal resection	1	4	0.013
Fistula	0	1	0.31
Perianal disease	7	3	1.0
Current smoking	3	1	1.0
Treatment			
5-aminosalicylates	11	4	0.31
Immunosuppressants	3	2	1.0
Antibiotics	1	3	0.063
Nutrition therapy	7	3	1.0
CDAI	183.9 (83.2–341.7)	197.7 (153.7–350)	0.61
CRP (mg/dL)	0.36 (0.07–8.18)	3.63 (0.02–5.75)	1.0

Parametric data are expressed as median (range).
CDAI, Crohn's disease activity index; CRP, C-reactive protein; N-group, no-remission group; R-group, remission group.

Table 2 Clinical course during the induction therapy by infliximab

	Week 0 (baseline)	Week 2	Week 6	Week 14
CDAI				
R-Group	183.9 (83.2–341.7)	73.3 (55.5–144)***	60.1 (29.1–97)***	59.4 (14.8–110.7)***
N-Group	197.7 (153.7–350)	155.7 (127.2–406.2)	165.4 (111–386.4)	195.2 (154.6–291.1)
CRP (mg/dL)				
R-Group	0.36 (0.07–8.18)	0.05 (0.01–0.45)**	0.04 (0.01–0.37)***	0.05 (0.01–2.6)*
N-Group	3.63 (0.02–5.75)	0.18 (0.01–0.65)	0.11 (0.01–5.03)	0.7 (0.01–3.87)

Data are expressed as median (range). Values are compared to those at week 0 (baseline) using Wilcoxon signed-rank test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.
CDAI, Crohn's disease activity index; CRP, C-reactive protein; IFX, infliximab; N-group, no-remission group; R-group, remission group.

subsequently identified 14 miRNAs that showed altered signals by twofold or more when compared between the samples of weeks 0 and 6. The relative values of 14 miRNAs at week 6 are listed in Table 3.

These miRNA levels were additionally measured by real-time PCR using the same samples in order to compare the values of array profiling. As shown in Figure 1, the values determined by two different methods showed an excellent agreement ($\rho = 0.838$, $P < 0.0001$).

Table 3 miRNAs showing altered expression during the induction therapy by infliximab

Increased miRNAs		Decreased miRNAs	
miRNA	Fold change	miRNA	Fold change
miR-361-5p	4.49	miR-324-3p	0.46
miR-127-3p	4.00	miR-486-3p	0.48
miR-28-5p	3.46		
miR-539	2.79		
miR-224	2.77		
let-7e	2.64		
miR-221	2.56		
miR-125a-5p	2.28		
miR-485-3p	2.27		
let-7d	2.23		
miR-199a-3p	2.11		
miR-223	2.02		

Data are shown as average fold change of miRNA levels of week 6 relative to those of week 0 ($n = 3$).

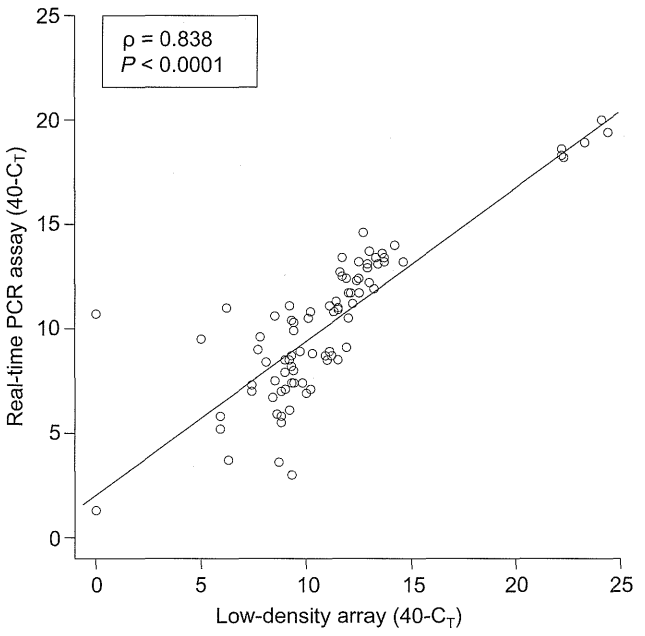

Figure 1 Comparison of cycle threshold (C_T) values by low-density array and real-time polymerase chain reaction (PCR). Fourteen candidate microRNAs (miRNAs) elicited by low-density array were additionally measured by real-time PCR using the same samples. Significantly positive correlation was found by Spearman's rank correlation coefficient.

Assessment of candidate miRNAs by real-time PCR. The 14 candidate miRNA levels were further analyzed by real-time PCR using the samples of the other 16 CD patients. When C_T values < 35 were regarded as positive, detection rates of miR-485-3p and miR-539 were low (72% and 28%, respectively). We thus excluded these two miRNAs from the following validation analysis. As shown in Figure 2, the relative values of five miRNAs (let-7d, let-7e, miR-28-5p, miR-221, and miR-224) at week 6 were significantly higher than those at week 0 ($P < 0.05$). There was a trend toward a higher value of miR-125a-5p at week 6 when compared with the value at week 0. However, the difference did not reach a statistical significance ($P = 0.051$). No obvious difference was observed between the values at weeks 0 and 6 in the remaining six miRNAs.

Correlation of miRNAs expression with inflammatory parameters at baseline. In order to ascertain possible correlation of miRNA levels with inflammatory parameters, we compared miRNA levels with CDAI values, serum CRP levels, white blood cell counts, and platelet counts at week 0. As shown in Table 4, neither CDAI values nor serum CRP levels showed significant correlation with miRNA levels at week 0. Other inflammatory parameters, including white blood cell counts and platelet counts, did not show any correlation with miRNA levels (data not shown).

Correlation of miRNA expression with therapeutic efficacy by IFX. We subsequently analyzed the incremental ratio of five miRNAs according to the therapeutic efficacy by IFX. As indicated in Figure 3A, the incremental ratio of let-7e was statistically significant in R-group when compared with that in N-group (median fold change: 1.68 *vs* 0.68, $P = 0.031$). The incremental ratio of let-7d tended to be higher in R-group than in N-group, whereas the difference did not reach a statistical significance (median fold change: 1.57 *vs* 0.83, $P = 0.089$).

Figure 3B shows the individual expression levels of let-7d and let-7e at weeks 0 and 6. Although significantly increased expression of let-7d and let-7e at week 6 were observed in R-group ($P = 0.001$ and 0.002, respectively), such a trend was not observed in N-group.

Discussion

Several factors associated with therapeutic effect of IFX have been rigorously investigated from clinical, immunological, and genetic viewpoints.[4–9] Consequently, some clinical biomarkers, including CRP level,[6] have been suggested to be possible candidates. However, because clinical biomarkers do not show similar trends in serial changes, it remains unclear whether they are inevitably reliable. In this regard, we investigated the association of miRNAs with therapeutic effect of IFX in CD. Considering miRNAs to have potential pathophysiological roles in various diseases, they are provisionally plausible targets for this kind of analysis.

In the present study, we investigated serum miRNA levels in CD patients, and detected five miRNAs (let-7d, let-7e, miR-28-5p, miR-221, and miR-224) that demonstrated significant increases during the induction therapy by IFX. Among them, two miRNAs (let-7d and let-7e) were shown to have a similar expression pattern

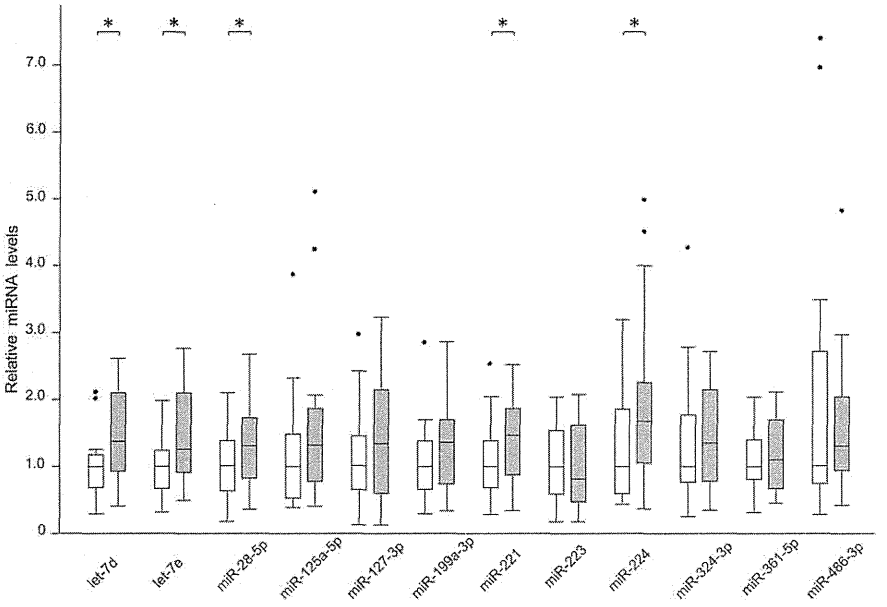

Figure 2 Relative microRNAs (miRNA) levels of week 0 and week 6 in validation cohort (n = 16). Relative expression levels of each miRNA are shown as ratio to the median value of week 0. Boxes indicate the interval between the 25th and 75th percentiles, and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 × IQR. miRNA expression levels of week 0 (white boxes) and week 6 (gray boxes) were compared by Wilcoxon signed-rank test. *$P < 0.05$.

Table 4 Correlation between miRNAs and clinical parameters at baseline

miRNA	CDAI		CRP	
	ρ	*P* value	ρ	*P* value
let-7d	0.17	0.53	0.37	0.16
let-7e	0.12	0.65	0.39	0.13
miR-28-5p	0.08	0.77	0.19	0.49
miR-221	0.22	0.42	0.14	0.61
miR-224	0.17	0.53	0.02	0.94

Correlation is assessed by Spearman's rank correlation coefficient. CDAI, Crohn's disease activity index; CRP, C-reactive protein.

according to the therapeutic effect of IFX. Because miRNA levels at baseline did not show any correlation with inflammatory parameters including CDAI and CRP, it can be possible that let-7d and let-7e are novel biomarkers closely associated with therapeutic efficacy of induction therapy by IFX.

Let-7d and let-7e are the members of the let-7 family that contain several miRNAs with high sequence homology.[27] Although biological functions of let-7 miRNAs have been reported to range widely,[28,29] recent studies have demonstrated a significant role of let-7 miRNAs in the regulation of apoptosis through the inhibition of Fas[30,31] and Bcl-xL.[32] It has been demonstrated that a decrease in Bax expression with concurrent higher Bcl-xL/Bax ratio occurs in the lamina propria T lymphocytes from CD patients, and that such imbalances of pro-apoptotic and anti-apoptotic Bcl-2 protein family contribute to resistance to apoptosis and to chronicity of inflammation.[33,34] Because the anti-inflammatory effect of IFX is partly attributed to the induction of apoptosis of lamina propria T lymphocytes[35–37] and monocytes,[38] and because IFX induces apoptosis of such effector immune cells through mediating the expressions of Bcl-2 protein family,[37–39] let-7 miRNAs seem to play a certain role via controlling apoptosis of effector immune cells. Considering their function for the inhibition of Bcl-xL, let-7 miRNAs may assist pro-apoptotic effect of IFX with the restoration of the balance of Bcl-2 protein family in inflammatory cells, thus resulting in positive association with therapeutic efficacy by IFX. In addition, 3′-untranslated region of IL-23R gene has been recently disclosed to induce loss of let-7e/f regulation and enhanced protein production in IBD patients.[40] Because a close association of IL-23R in the pathogenesis of CD has been demonstrated from genetic[41] and immunological aspects,[42] an association between IL-23R and let-7e/f further imply the contribution of let-7 miRNAs in CD.

miR-28-5p, miR-221, and miR-224 increased significantly during the induction therapy by IFX. However, we failed to demonstrate positive association of those miRNAs with the therapeutic efficacy by IFX. Among these miRNAs, the upregulation of miR-221 has been reported to have an association with liver fibrosis. Furthermore, possible contribution of miRNAs to intestinal fibrosis of CD has been recently reported.[43] Since the induction therapy by IFX can cause dramatic mucosal healing of inflamed intestine, altered expression of miR-221 during the induction therapy by IFX may be representative of the regenerative process of luminal CD. In addition, an increase in the expression of miR-28-5p has been shown in blood samples from UC patients.[44,45] It thus can be possible that these miRNAs are subclinical biomarkers predictive of the therapeutic efficacy of IFX.

In addition to the identification of 11 miRNAs as possible diagnostic biomarkers in pediatric CD patients, Zahm *et al.* also showed decreases in the expression of several miRNAs after medical treatments for 6 months.[25] However, we failed to show any association of those miRNAs with the efficacy of IFX as the induction therapy. Such contradictory results may be caused by the following two reasons. First, the subjects recruited by Zahm *et al.* were pediatric CD patients, who generally manifest widespread involvement and rapid disease progression when compared with adult CD patients.[46,47] Furthermore, growth failure may have altered the pattern of miRNA expressions in such pediatric

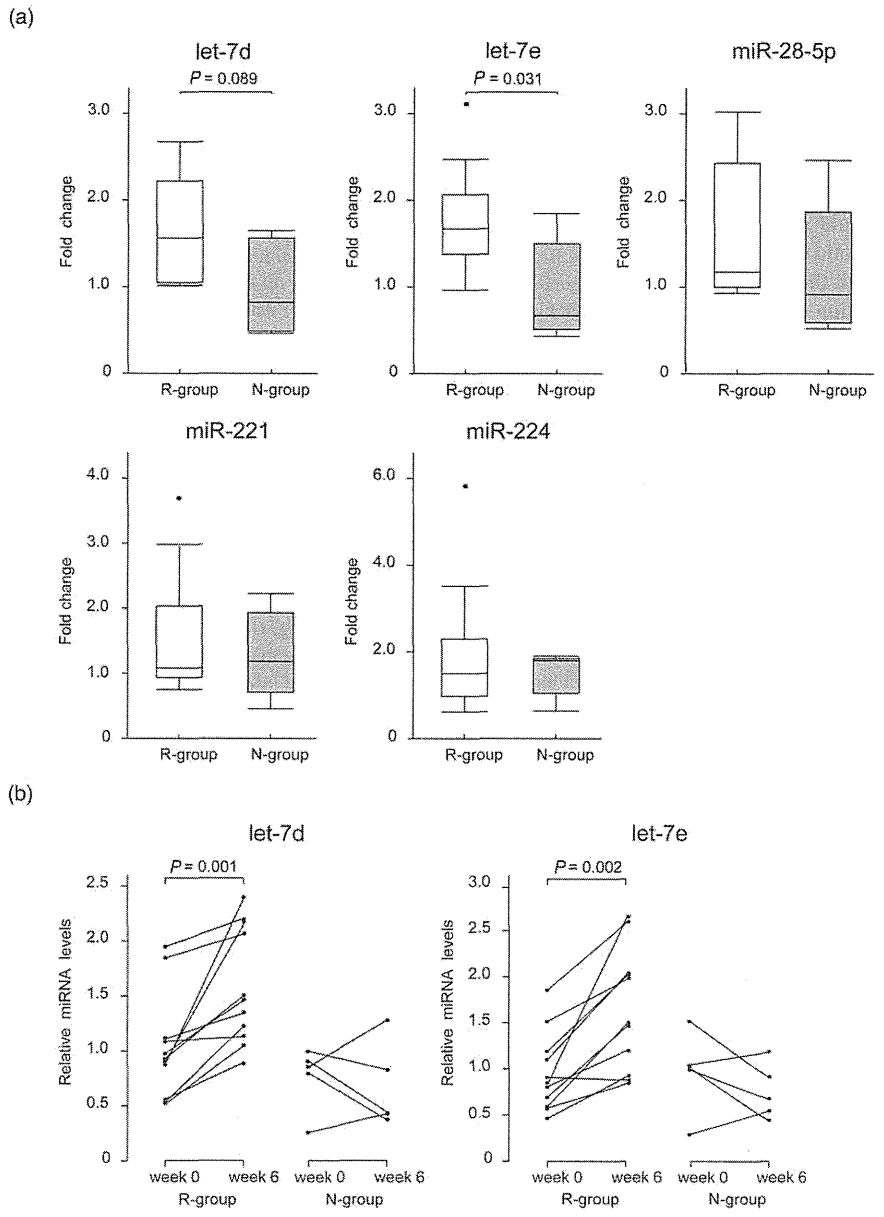

Figure 3 Relative change of microRNAs (miRNA) levels according to therapeutic efficacy by infliximab (IFX). (a) Comparison of relative change of miRNA levels between R-group and N-group. Data are shown as ratio of miRNA levels of week 6 relative to those of week 0. Boxes indicate the interval between the 25th and 75th percentiles, and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 × interquartile ranges (IQR). Closed circles indicate data points outside 1.5 × IQR. Mann–Whitney *U*-test identified significantly greater change of let-7e in R-group than in N-group. (b) Comparison of let-7d and let-7e levels of week 0 and week 6 in R-group and N-group. Wilcoxon singed-rank test showed significantly higher levels of let-7d and let-7e of week 6 when compared with the values of week 0 in R-group.

CD patients. Second, since we aimed to identify miRNAs in association with therapeutic efficacy of IFX, the nomination of candidate miRNAs was methodologically different from that of the previous investigation. It thus seems likely that miRNAs identified in the present study are distinctive with respect to clinical implication and they are specific to the therapeutic efficacy of IFX in adult CD patients.

The present study has several limitations. First, we failed to validate twofold or more altered expression of nine miRNAs (miR-125a-5p, miR-127-3p, miR-199a-3p, miR-223, miR-324-3p, miR-361-5p, miR-485-3p, miR-486-3p, and miR-539) that were initially elicited by low-density array. A possible explanation of the inconsistent results would be the small number of subjects allocated for both analyses. Another source of the inconsistency might be a relatively small amount of extracted RNAs especially for low-abundance targets, although miRNA levels determined by low-density array and real-time PCR showed excellent agreements (Fig. 1). On the other hand, since let-7d and let-7e demonstrated sufficient levels of signal intensity (range of C_T values: from 25 to 29), we believe that the significant association of these miRNAs with therapeutic efficacy of IFX is reliable. Second, the number of enrolled patients was small so that some of the patients' demographics were significantly different between our R-group and N-group. Because our present study was a pilot one, a well-designed study with a large sample size is mandatory to explore valid biomarkers predictive of therapeutic effect of IFX. Third, we did not measure miRNA levels in healthy subjects. Accordingly, the increased let-7d/e levels in R-group do not necessarily indicate the augmentation of those miRNA functions. However, considering the significant increase in let-7d/e levels in R-group compared