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ORIGINAL PAPER

Exact break point of a 50 kb deletion 8 kb centromeric of the *HLA-A* locus with *HLA-A*24:02*: the same deletion observed in other *A*24* alleles and *A*23:01* allele

Shigeki Mitsunaga · Yuko Okudaira · Nanae Kunii · Tailin Cui · Kazuyoshi Hosomichi · Akira Oka · Yasuo Suzuki · Yasuhiko Homma · Shinji Sato · Ituro Inoue · Hidetoshi Inoko

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Abstract In a structural aberration analysis of patients with arthritis mutilans, a 50 kb deletion near the *HLA-A* locus with *HLA-A*24:02* allele was detected. It was previously reported that *HLA-A*24:02* haplotype harbored a large-scale deletion telomeric of the *HLA-A* gene in healthy individuals. In order to confirm that the deletion are the same in patients with arthritis mutilans and in healthy individuals, and to identify the break point of this deletion, the boundary sequences across the deletion in *A*24:02* was amplified by polymerase chain reaction (PCR) as a 3.7 kb genomic fragment and subjected to nucleotide sequence determination. A comparison of these genomic sequences with those of the non-*A*24:02* haplotype revealed that the deleted genomic region spanning 50 kb was flanked by 3.7 kb repetitive element-rich segments homologous to each other on both sides in non-*A*24*. The nucleotide sequences of the PCR products were identical in patients with arthritis mutilans and in healthy individuals, revealing that the deletion linked to *A*24:02* is irrelevant to the onset of arthritis mutilans. The deletion was detected in all other *A*24* alleles so far examined but not in other *HLA-A* alleles, except *A*23:01*. This finding, along with the phylogenic tree of *HLA-A* alleles and the presence of the 3.7 kb highly homologous segments at the boundary of the deleted genomic region in *A*03* and *A*32*, may suggest that this *HLA-A*24:02*-linked deletion was generated by homologous recombination within two 3.7 kb homologous segments situated 50 kb apart in the ancestral *A*24* haplotype after divergence from the *A*03* and *A*32* haplotypes.

Keywords *HLA-A*24* · Deletion · Arthritis mutilans · Evolution

S. Mitsunaga · Y. Okudaira · N. Kunii · T. Cui · K. Hosomichi · A. Oka · I. Inoue · H. Inoko (✉)
Department of Molecular Life Sciences, Division of Basic Medical Science and Molecular Medicine,
Tokai University School of Medicine,
143 Shimokasuya, Isehara,
Kanagawa 259–1193, Japan
e-mail: hinoko@is.icc.u-tokai.ac.jp

Y. Suzuki · S. Sato
Department of Internal Medicine,
Tokai University School of Medicine,
143 Shimokasuya, Isehara,
Kanagawa 259–1193, Japan

Y. Homma
Department of Clinical Health Science,
Tokai University School of Medicine,
143 Shimokasuya, Isehara,
Kanagawa 259–1193, Japan

Introduction

The disease concordance rate of rheumatoid arthritis is about 12–15% in monozygotic twins, 3–4% in dizygotic twins, and 2–4% in non-twin siblings (Silman et al. 1993). The ratio of the risk of disease recurrence among the siblings of affected individuals to disease incidence in the general population (λ) is 5–10 (Wandstrat and Wakeland 2001). Therefore, the contribution of genetic predisposition to RA onset is relatively large. Arthritis mutilans is a very

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severe rheumatoid arthritis and shows very destructive changes as a result of joint space narrowing, erosions, resorptive changes, and subluxation (Ochi et al. 1988). Expecting genome structural aberration, large deletions/insertions, and copy number variation due to the severe symptoms of arthritis mutilans, we conducted a structural aberration analysis using comparative genomic hybridization (CGH) array. A 50 kb deletion near the *HLA-A* locus was detected by the CGH array analyses in Japanese patients with arthritis mutilans. In the following analysis, 11 out of 12 patients with arthritis mutilans was found to have *A*24:02* allele, suggesting a possibility that this deletion may be linked to *HLA-A*24:02*, irrespective of the presence or absence of the disease.

The *HLA-A* locus in B-cell lines and peripheral leukocytes bearing genetically defined *A*24:02*, as well as serologically defined A24 and A23, was previously reported to accompany a large-scale deletion (el Kahloun et al. 1992; Geraghty et al. 1992; Venditti and Chorney 1992; Watanabe et al. 1997). So far, however, neither the break point nor the boundary sequences have been identified. It is very interesting to investigate whether or not the deletion near the *HLA-A* locus with *A*24:02* in arthritis mutilans is identical to that in healthy individuals and to determine when this deletion was generated in the evolutionary process of the *A*24* and its related allelic groups. Therefore, in the present study, we analyzed boundary sequences of the *A*24*-linked deletion by long-range polymerase chain reaction (PCR) followed by nucleotide sequence determination and a comparison between arthritis mutilans and healthy individuals. Based on this sequence information, we also analyzed the presence or absence of this deletion in the *A*24* allele and its related allele groups by duplex PCR.

Materials and methods

Subjects

Twelve Japanese patients with arthritis mutilans were enrolled from among outpatients of the Division of Rheumatology, Tokai University Hospital (Table 1). A total of 2,013 unrelated healthy Japanese control subjects were recruited from among visitors to the Health Evaluation and Promotion Center of Tokai University Hospital. All of the subjects gave written informed consent for genetic screening. Genomic deoxyribonucleic acids (DNAs) carrying *A*23:01*, which were distributed by the UCLA International Cell Exchange, were kindly gifted by Dr. Koichi Kashiwase of the Japanese Red Cross Tokyo Blood Center. The study was approved by the Ethics Committee of Tokai University School of Medicine.

Table 1 *HLA* alleles in arthritis mutilans

ID	HLA-A		HLA-B		DRB1	
RA_M1	**24:02*		**52:01*	**59:01*	**04:05*	**15:02*
RA_M2	**24:02*	**26:02*	**40:01*	**40:06*	**04:05*	**09:01*
RA_M3	**24:02*		**52:01*	**59:01*	**04:05*	**15:02*
RA_M4	**24:02*		**35:01*	**40:02*	**09:01*	**15:01*
RA_M5	**02:01*	**24:02*	**35:01*	**52:01*	**09:01*	**15:02*
RA_M6	**11:01*	**24:02*	**48:01*	**54:01*	**04:05*	**09:01*
RA_M7	**24:02*		**40:02*	**52:01*	**04:05*	**15:02*
RA_M8	**11:01*	**24:02*	**40:01*	**40:06*	**04:05*	**09:01*
RA_M9	**24:02*	**33:03*	**44:03*	**51:01*	**09:01*	**15:02*
RA_M10	**24:02*	**31:01*	**07:02*	**35:01*	**01:01*	**14:01*
RA_M11	**02:06*	**24:02*	**15:11*	**48:01*	**04:05*	**04:07*
RA_M12	**02:01*	**26:03*	**40:01*	**51:01*	**09:01*	**12:01*

Analysis of structural aberration and HLA typing

DNA extracted from peripheral blood using the conventional method was employed for the following analyses. The Human Genome CGH microarray kit (244 K) was used on a Hybridization Oven G2545A and a DNA Microarray Scanner G2565BA according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA) for the analysis of structural aberration. *HLA-A*, *HLA-B*, and *HLA-DRB1* allele typing was performed using the Luminex assay system and human leukocyte antigen (HLA) typing kits (WAKFlow HLA typing kit, Wakunaga, Osaka, Japan or LABType SSO, One Lambda, Canoga Park, CA, USA).

Polymerase chain reactions

The PCR primers used in this study are listed in Table 2, and their positions in the HLA-A gene region are shown in Fig. 1a. Long-range PCR for amplifying a 3,677 bp genomic segment that included the boundary region across the deletion near the *HLA-A* locus with *HLA-A*24* was performed in a 25 μl aliquot using 320 nM PCR primers, Adel_F2 and Adel_R2 (Table 2), 32 ng of genomic DNA, 0.5 U of KOD FX polymerase (Toyobo, Osaka, Japan), 0.4 mM each of dNTP and 2× PCR buffer for KOD FX (Toyobo). The PCR thermal conditions were as follows: 94°C for 2 min; 5 cycles consisting of denaturation (98°C, 10 s), annealing, and extension (72°C, 5 min); 5 cycles consisting of denaturation (98°C, 10 s), annealing, and extension (70°C, 5 min); 5 cycles consisting of denaturation (98°C, 10 s), annealing, and extension (68°C, 5 min); 20 cycles consisting of denaturation (98°C, 10 s), annealing, and extension (66°C, 5 min); and 66°C for 7 min. Long-

Table 2 Primers used in this study

Primer name	Sequence 5′-3′	Position in NC_000006.11
Adel_ F2	5′-CAGTGAACAGACATCCTACAGAATGGGAGA-3′	29850471-29850500
Adel_ R2	5′-CCCATGACCTGCTACGTAACTGTTCTTTTC-3′	29908962-29908991
A+F1	5′-CTATAATATCGCCCTCCCTCTGGTCTTGAG-3′	29856608-29856637
A+R1	5′-TGTTGTCCAATCCTATCCCCTAAAAGGAAC-3′	29861622-29861651

range PCR products were subjected to direct nucleotide sequence determination. To discriminate *A*24*-linked deletion-negative DNAs from deletion-positive ones by PCR, a PCR primer pair, A+F1 and A+R1 (Table 2), was designed from the genome sequence within the deleted region linked to *HLA-A*24* (Fig. 1a), giving a 5,044 bp PCR band. The PCR thermal conditions are the same as described above.

Analyses of nucleotide sequences

The ClustalW program (Thompson et al. 1994; http://clustalw.ddbj.nig.ac.jp/top-j.html) was used for construction of a phylogenic tree. Dot plots were obtained by the program UGENE (http://ugene.unipro.ru/) using the following parameters: minimum repeat length 50 bp; repeat identity 100%.

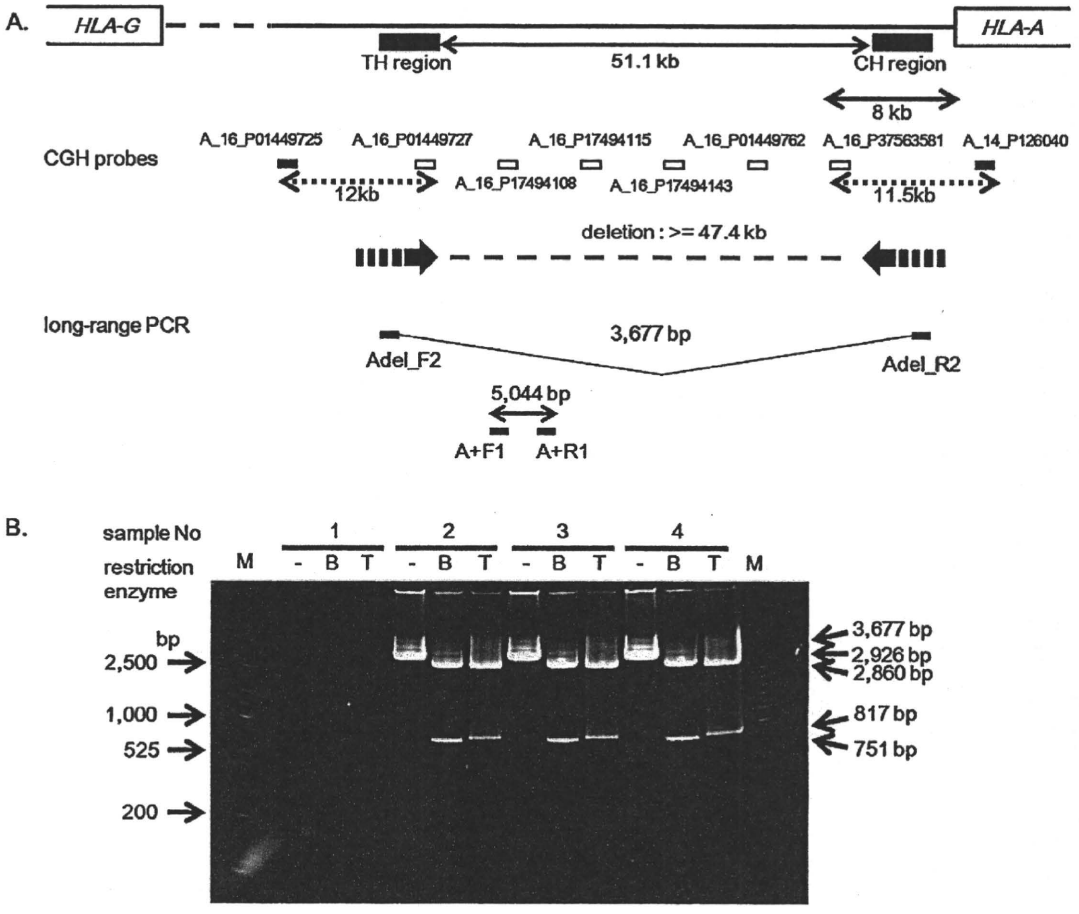

Fig. 1 PCR amplification of the boundary region across the deletion near the HLA-A locus with the *HLA-A*24:02* allele. **a** Schematic illustration of the deleted region around the HLA-A gene. *Closed boxes*: Positive probes in the array CGH analysis of *A*24:02*. *Open boxes*: Negative probes in the array CGH analysis of *A*24:02*. The boundary region with a mosaic fragment between the TH and CH regions, which are highly homologous to each other, was amplified across the break point in long-range PCR using the Adel_F2 and Adel_R2 primers from *A*24:02*. **b** SDS-PAGE patterns of the PCR products using the primers Adel_F2 and Adel_R2. – The absence of the restriction enzyme, *B* *Bsr*DI digest, *T* *Taq*I digest, *M* DNA size marker 50–2,500 bp ladder

Results

Structural aberration analysis of arthritis mutilans using CGH microarray indicated that the probes A_16_P01449725 (nucleotide positions 29842827–29842885 near the *HLA-A* gene in the HLA region on chromosome 6 of GRCh37 reference assembly NC_000006.11) and A_14_P126040 (nucleotide positions 29913915–29913965) gave positive signals, but that all five probes located between them, A_16_P01449727 (nucleotide positions 29854870–29854929) and A_16_P37563581 (nucleotide positions 29902255–29902314), gave negative signals (Fig. 1a). This fact may suggest that a genomic region is absent in patients with arthritis mutilans; this region is at least 47,445 bp in length, between the probes A_16_P01449727 and A_16_P37563581 (Fig. 1a) just 8 kb telomeric of the HLA-A gene. By *HLA-A* allele typing, 11 out of 12 patients with arthritis mutilans were found to have *HLA-A*24:02* (Table 1). All of these 11 samples of *HLA*A*24:02*-positive arthritis mutilans gave negative signals by the probes between A_16_P01449727 and A_16_P37563581 in the CGH microarray analysis, whereas the remaining *A*24:02*-negative ones gave positive signals by the probes between A_16_P01449727 and A_16_P37563581. Since a large scale of genomic deletion near the *HLA-A* locus with *HLA-A*24* and *HLA-A*23* was previously reported (el Kahloun et al. 1992; Geraghty et al. 1992; Venditti and Chorney 1992; Watanabe et al. 1997), the deletion that we detected in the CGH microarray analysis is probably not specific to patients with arthritis mutilans but generally present in all *HLA-A*24*-positive individuals including healthy ones.

To precisely determine the break point near the *HLA-A* locus with *HLA-A*24:02*, we developed a long-range PCR using a pair of primers, Adel_F2 and Adel_R2, to amplify the boundary region across the deletion, as described in Materials and Methods (Table 2 and Fig. 1b). Nucleotide sequences of the PCR products obtained from two patients with arthritis mutilans with *HLA-A*24:02* and also from two healthy individuals with *HLA-A*24:02* were determined (accession number AB603755). Accordingly, it was found that the PCR product was 3,677 bp in length and that the nucleotide sequences were identical among both patients and healthy individuals. The mobility of the fragments obtained after *Bsr*DI digestion or *Taq*I digestion of the PCR product on SDS polyacrylamide gel electrophoresis showed good concordance with those calculated from the nucleotide sequences, (2,926 bp+751 bp) for *Bsr*DI and (2,860 bp+817 bp) for *Taq*I (Fig. 1b). The nucleotide sequences around the break point are quite rich in repetitive sequences, as assessed by RepeatMasker (Smit et al.) analysis (Table 3).

We developed a duplex long-range PCR system to screen this deletion near the *HLA-A* locus in a large number of samples. In this system, PCR primers for amplifying deletion-positive samples are identical to the primers used in the above experiments, and the PCR primers (A+F1 and A+R1) for amplifying deletion-negative samples were designed from the genomic sequences deleted in HLA-A*24-positive individuals (Table 2 and Fig. 1a). The deletion-positive and deletion-negative samples could be clearly discriminated by the different lengths of PCR products obtained using this duplex PCR system (Fig. 2). All of the *A*24:02* individuals from both the patients with arthritis mutilans and the healthy individuals were found to carry the deletion, giving PCR products with 3.7 kb in length, as expected.

In this duplex long-range PCR system, all of the *HLA-A* alleles so far examined, other than those in the *HLA-A*24* allele group, gave PCR products of 5.0 kb in length except *HLA-A*23:01*, revealing the absence of deletion. Further, all of the alleles in the *A*24* allele group, such as *A*24:04*, *A*24:07*, *A*24:08*, and *A*24:20* alleles, gave the 3.7 kb PCR products as in *A*24:02*, indicating the presence of this 50 kb deletion in the *A*24* allele

Table 3 Repetitive sequences in the boundary region across the deletion: RepeatMasker analysis of the PCR product from RA-M7

Position in RA-M7		Strand	Percent Div.	Position in repeat		Name	Family	Class
Begin	End			Begin	End			
2	963	+	8.9	5193	6163	L1PA10	L1	LINE
1052	1150	+	22.0	6	103	MLT1F1	ERVL-MaLR	LTR
1151	1532	+	14.2	1	375	THE1C	ERVL-MaLR	LTR
1533	1977	+	22.0	104	561	MLT1F1	ERVL-MaLR	LTR
2055	2334	-	21.8	1	287	AluJo	Alu	SINE
2340	2488	+	31.1	2230	2389	Charlie9	hAT-Charlie	DNA
2498	2783	+	23.1	3	302	MLT1E3	ERVL-MaLR	LTR
2784	3090	+	9.4	1	304	AluY	Alu	SINE
3091	3347	+	23.1	303	573	MLT1E3	ERVL-MaLR	LTR
3581	3629	-	20.4	2813	2865	L2	L2	LINE

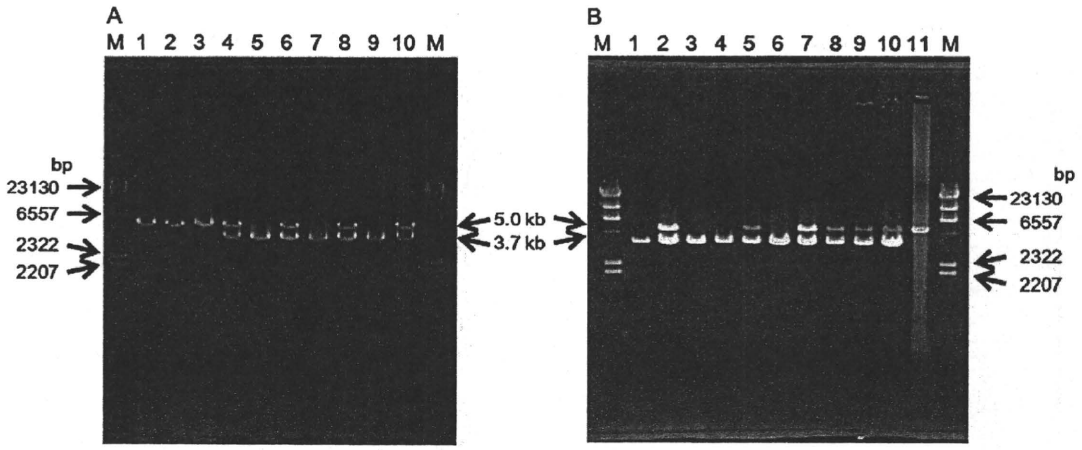

Fig. 2 Duplex PCRs using two PCR primer pairs discriminating between the deletion-positive and deletion-negative genomic DNAs. Smaller bands correspond to the PCR product from the haplotype carrying the deletion, and larger bands correspond to the haplotype not carrying the deletion. **a** SDS-PAGE pattern of duplex PCR product using healthy individual DNAs. *M* Molecular size marker, λ-DNA/*Hin*dIII digest; *1 A*02:07, A*33:03*; *2 A*02:06, A*02:07*; *3 A*26:02, A*33:03*; *4 A*24:02, A*31:01*; *5 A*24:02* homozygote; *6 A*24:02, A*16:01*; *7 A*24:02* homozygote; *8 A*24:02, A*26:01*; *9 A*24:02* homozygote; *10 A*02:01, A*24:02*. **b** SDS-PAGE pattern of duplex PCR products using arthritis mutilans DNAs. *M* Molecular size marker, λ-DNA/*Hin*dIII digest; *1 A*24:02* homozygote; *2 A*24:02, A*26:02*; *3 A*24:02* homozygote; *4 A*24:02* homozygote; *5 A*11:01, A*24:02*; *6 A*24:02* homozygote; *7 A*11:01, A*24:02*; *8 A*24:02, A*33:03*; *9 A*24:02, A*31:01*; *10 A*02:06, A*24:02*; *11 A*02:01, A*26:03*

haplotype (Fig. 3). A phylogenic tree constructed by the neighbor-joining (NJ) method (Saitou and Nei 1986) using the nucleotide sequences of exons 2 and 3 of the *HLA-A* alleles indicated that the deletion near the *HLA-A* gene was generated after the birth of *A*24* diverged from the *A*02* family (Fig. 4). It is notable that *A*23:01*, harboring this deletion, was generated after this divergence of the *A*24* allele group.

To identify the precise break point, we compared the nucleotide sequences of long-range PCR products obtained from the patient with *HLA-A*24:02* homozygous arthritis mutilans (RA-M7 in Table 1) carrying the deletion with those of the genomic region between the *HLA-A* and *HLA-G* genes from 12 genomic clones without this deletion, which have been deposited in GenBank: NT_113891.2 (*A*01:01*), NT_167244.1 (*A*01:01*), AC_000138.1

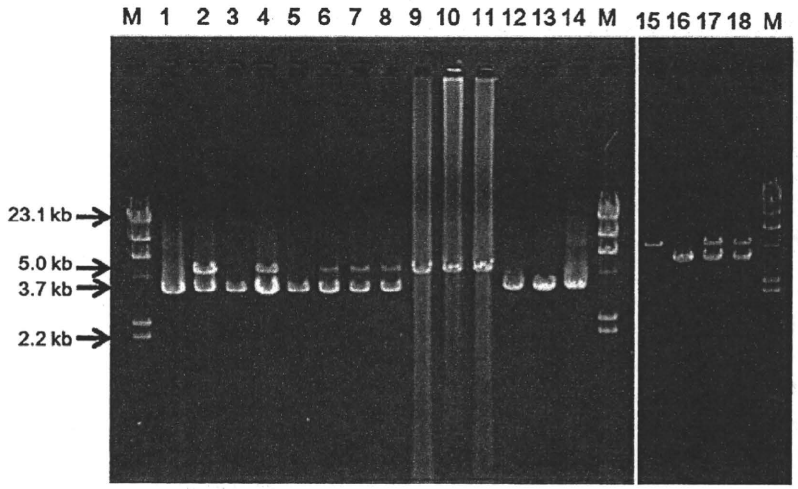

Fig. 3 The deletion related to *A*24:02* observed in the *A*24* and *A*23* allele groups. All genomic DNAs were obtained from healthy individuals. *M* DNA size marker, λ-DNA/*Hin*dIII digest; *1 A*24:02* homozygote; *2 A*24:02, A*31:01*; *3 A*24:02, A*24:20*; *4 A*02:02, A*24:20*; *5 A*24:02, A*24:08*; *6 A*02:06, A*24:08*; *7 A*01:01, A*24:02*; *8 A*01:01, A*24:02*; *9 A*02:01, A*26:02*; *10 A*01:01, A*02:06*; *11* A*26:01, *A*32:01*; *12 A*24:02, A*24:04*; *13 A*24:02, A*24:04*; *14 A*24:02, A*24:07*; *15 A*02:07, A*11:01*; *16 A*24:02* homozygote; *17 A*01:01, A*23:01*; *18 A*02:02, A*23:01*

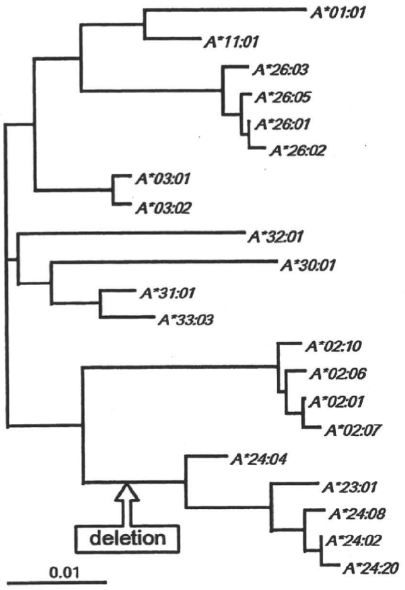

Fig. 4 A phylogenic tree of *HLA-A* alleles constructed by the neighbor-joining (NJ) method using the sequences of exons 2 and 3. It was noted that all of the *A*24* alleles, such as *A*24:02*, *A*24:04*, *A*24:07*, *A*24:08*, *A*24:20*, and *A*23:01*, are deletion-positive, and all of the alleles in groups other than *A*24*, such as *A*01:01*, *A*11:01*, *A*02:01*, *A*02:02*, *A*02:06*, *A*02:07*, *A*02:10*, *A*26:01*, *A*26:02*, *A*26:03*, *A*31:01*, and *A*33:03*, are deletion-negative as assessed by duplex long-range PCR

(*A*02:01*), NT_167245.1 (*A*02:01*), NW_001838980.1 (*A*02:01*), NC_000006.11 (*A*03:01*), NT_007592.15 (*A*03:01*), NT_167248.1 (*A*26:01*), AC_000049.1 (*A*29:02*), NT_167246.1 (*A*29:02*), NW_923073.1 (*A*29:02*), and NT_167249.1 (*A*32:01*). Two highly homologous sequences around the break point in the RA-M7 (*HLA-A*24:02* homozygote) sequences were located at the upstream of the *HLA-A* locus, physically 51.1 kb apart from each other (Fig. 1a). The centromeric homologous sequence (the one closer to the *HLA* locus), designated CH, is located 1.3 kb away from the *HLA-A* locus, and the telomeric one (the one more distant from the *HLA-A* locus), designated TH, is located 56.1 kb away from the *HLA-A* locus and 51.6 kb away from the *HLA-G* locus (Fig. 1a).

A dot plot was drawn to compare the genomic sequence of RA-M7 (*HLA-A*24:02* homozygote) with those of the 12 genomic clones described above with respect to the TH and CH regions using the program UGENE. The TH region from *HLA-A*03:01* (NC_000006.11) showed the highest similarity to the corresponding region of *HLA-A*24:02* among 12 genomic clones, and the CH region from *HLA-A*32:01* (NT_167249.1) showed the highest similarity to the corresponding region of *HLA-A*24:02*. Dot plots of the TH region from *HLA-A*03:01* and of the CH region from *HLA-A*32:01* against the nucleotide sequence of the long-range PCR product of RA-M7 (*HLA-A*24:02* homozygote) are shown in Fig. 5. The segments of repetitive sequences in the RA-M7 sequence, which were identified by Repeat Masker analysis, are also shown in Fig. 5. MLT1F1 and MLT1F3 were interrupted by THE1C and AluY, respectively (Fig. 5 and Table 3). The telomeric half of the boundary region across the deletion in *HLA-A*24:02*, which consisted of L1PA10, MLT1F1, THE1C, and MLT1F1, showed higher similarity to the telomeric half of the TH region of the *HLA-A*03:01* sequence than that of the *HLA-A*32:01* sequence. On the other hand, the centromeric half of the boundary region of *HLA-A*24:02*, which consisted of MLT1F1, AluJo, Charlie9, MLT1E3, AluY, MLT1E3, and L2, showed higher similarity to the

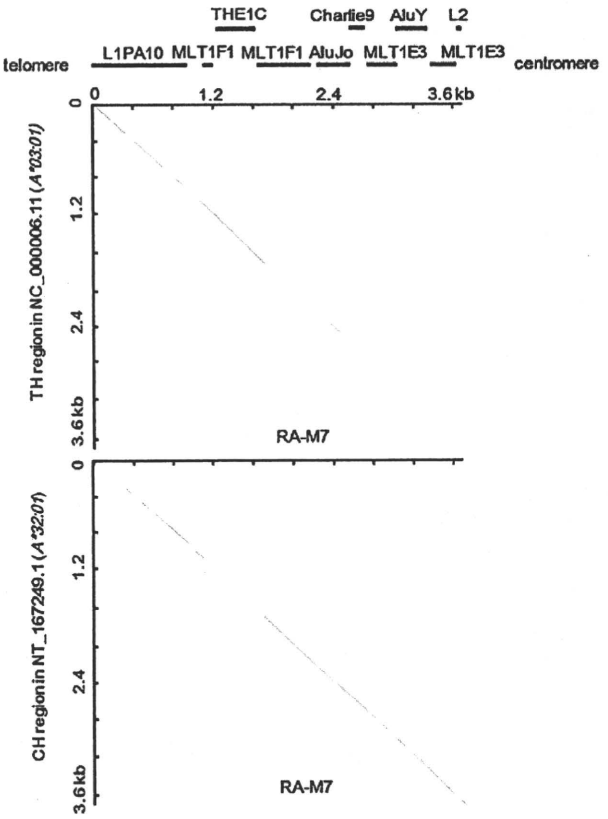

Fig. 5 Comparison of nucleotide sequences of the long-range PCR product from RA-M7 (*HLA*24:02* homozygote), which contains the boundary regions across the deletion, with those of *HLA-A*03:01* (NC_000006.11) and those of *HLA-A*32:01* (NT_167249.1). The *upper dot plot* was drawn by the comparison of RA-M7 with the TH region of *HLA-A*03:01* (NC_000006.11). The *lower dot plot* was drawn by the comparison of RA-M7 with the CH region of *HLA-A*32:01* (NT_167249.1). Repetitive sequence names and their positions in RA-M7, which were analyzed by RepeatMasker, are indicated *above the dot plots*

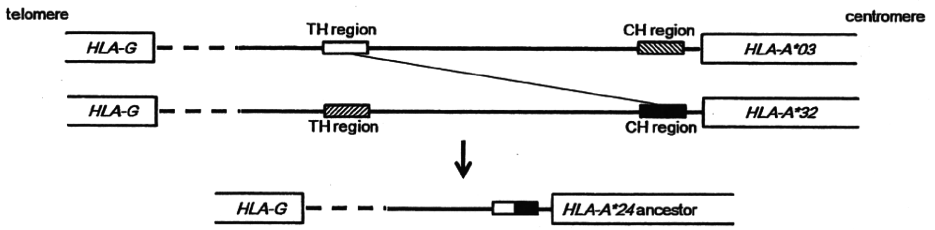

Fig. 6 Schematic illustration model for genetic recombination explaining the generation of the deletion near the *HLA-A* locus with the *A*24* and *A*23* haplotypes. A single-crossover event between the TH region of the *HLA-A*03* haplotype and the CH region of the *HLA-A*32* haplotype resulted in the generation of the deletion

centromeric half of the CH region of *HLA-A*32:01* than to that of the CH region of *HLA-A*03:01*. Along with this fact, a 23 bp sequence, AAGCAGCCCTGCAGGAAGGTCCA, in the MLT1F1 repeated sequence is shared by the TH region of *HLA-A*03:01* and the CH region of *HLA-A*32:01*, implying that genetic recombination between the (ancestral) *A*32* and *A*03*haplotypes occurred within this 23 bp sequence in MLT1F1, resulting in the generation of the 50 kb deletion after the divergence of the ancestral A*24 haplotype from the *A*03* and *A*32* allele groups.

Discussion

Although a large deletion near the *HLA-A* gene with A24 serotype or *A*24:02* allele was discovered in the 1990s by several groups (el Kahloun et al. 1992; Geraghty et al. 1992; Venditti and Chorney 1992; Watanabe et al. 1997), the break point has not been identified to date. We determined the precise position of the break point using a long-range PCR for amplifying the boundary region across the break point. The nucleotide sequences of the PCR products with 3.7 kb in length obtained from the *A*24:02* homozygotes are quite rich in repetitive sequence elements. In addition, we found two highly homologous sequences about 50 kb apart each other in the deletion-negative HLA-A haplotypes harboring other than *A*24* and *A*23* alleles by comparison of the break point boundary sequences with the deletion-negative haplotypes. We designated the two homologous sequences as TH region for more telomeric region from HLA-A gene and CH region for more centromeric region to HLA-A gene, respectively. The existence of two homologous sequences, TH and CH regions, in the boundary region of the break point suggests that the deletion was generated by the homologous recombination (single crossover) between TH and CH regions. The duplex long-range PCR showed that not only *A*24:02* and *A*23:01* but also other *A*24* alleles, such as *A*24:04*, *A*24:07*, *A*24:08*, and *A*24:20* alleles, have the 50 kb deletion. Therefore, it was suggested that these alleles diverged from the ancestral *A*24/A*23* allele after the homologous recombination between TH and CH regions.

In the nucleotide sequence comparison by dot plots, the long-range PCR product of RA-M7 (homozygote of *A*24:02*) showed higher similarity with the TH region from *HLA-A*03:01* (NC_000006.11) in the telomeric half and showed higher similarity with the CH region from *HLA-A*32:01* (NT_167249.1) in the centromeric half. In addition, the telomeric half of the TH region of *HLA-A*03:01* (NC_000006.11) and the centromeric half of *HLA-A*32:01* (NT_167249.1) overlapped with a 23 bp sequence, AAGCAGCCCTGCAGGAAGGTCCA, in the MLT1F1 segment. Collectively, these observations suggest that the 50 kb deletion near the *HLA-A* locus with *HLA-A*24* and *HLA-A*23* was generated by the homologous recombination within the 23 bp sequence between the TH region of the *A*03:01* haplotype and the CH region of the *A*32:01* haplotype (Fig. 6). Interestingly, the nucleotide sequence of the second exon of *A*32:01* is more similar to that of *A*24:02* than that of *A*02*, which is the closest to *A*24* in the phylogenic tree drawn using the exons 2 and 3 nucleotide sequences (Fig. 4). This fact supports the hypothesis that genetic recombination had a role in generating the deletion near the *HLA-A* locus described above.

The nucleotide sequence of exon 3 of *A*24:02* is more similar to that of *A*02:01* than to that of *A*32:01*. Therefore, it is likely that the second single crossover occurred around the region from intron 2 to exon 3 between the ancestral *A*24* and *A*02* haplotypes either after or before the generation of the deletion near the HLA-A locus with the ancestral *A*24* and *A*23* haplotypes (Fig. 6). Alternatively, the double-crossover event (Chen et al. 2007, Blanco et al. 2000) between the *A*32:01* and *A*02:01* haplotypes could provide another model for the generation of the deletion and also the birth of the common ancestor of the *A*24* and *A*23* haplotypes. Further analysis of genomic

sequencing of the related HLA haplotype is required for the evaluation of this alternative model.

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Conflicts of interest None

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Particular human leukocyte antigen alleles are associated with biochemical traits in the Japanese population

Shigeki Mitsunaga [a], Yasuhiko Homma [b], Akira Narita [a], Koichi Kashiwase [c], Yuko Okudaira [a], Yutaka Shiina [b], Ituro Inoue [a], Hidetoshi Inoko [a,*]

[a] Department of Molecular Life Sciences, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Kanagawa, Japan
[b] Department of Clinical Health Science, Tokai University School of Medicine, Kanagawa, Japan
[c] Department of Laboratory, Japanese Red Cross Tokyo Blood Center, Tokyo, Japan

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ABSTRACT

We analyzed genetic associations among 7 biochemical traits (fasting plasma glucose, HbA1c, total cholesterol, low-density lipoprotein [LDL] cholesterol, high-density lipoprotein cholesterol, triglyceride, and uric acid) and 6 *HLA* loci using 1,616 individuals who visited the Health Evaluation and Promotion Center at Tokai University Hospital. Significant differences between the individuals carrying particular *HLA* alleles and those not carrying the alleles in certain biochemical traits were observed by Mann–Whitney *U* test. In female subjects, *DPB1*03:01* was significantly associated with HbA1c ($p = 0.0000665$), and *DRB1*14:03* was associated with total cholesterol concentration ($p = 0.0015$). In male subjects, *C*14:02* demonstrated significant associations with fasting plasma glucose with *p* values of 0.0041. By contrast, Fisher's exact test indicated that female *DRB1*14:03* was associated with a high concentration of total cholesterol ($p = 000323$, odds ratio [OR] = 4.32, 95% confidence interval [95% CI] = 1.83–10.36), whereas female *DPB1*02:01* had a protective effect against a high concentration of LDL cholesterol ($p = 0.0043$, OR = 0.41, 95% CI = 0.19–0.79). These associations have a statistical power of more than 0.8 and still retain significance after Bonferroni correction.

1. Introduction

Human leukocyte antigens (HLA) play a pivotal role in the immune system by presenting self-peptides or non-self-peptides to helper and cytotoxic T cells. Presenting non-self-peptides derived from pathogens on HLA, professional antigen-presenting cells such as dendritic cells, and macrophages activate T cells. Activated T cells and antigen-presenting cells secrete various kinds of cytokines, including proinflammatory cytokines. These activation and cytokine secretions result in clonal expansions of T cells, cytolysis of target cells, and antibody production. The range of these reactions is precisely controlled through the activation of T-cell clones, which are specifically reactive to the complex of peptide and HLA. Self-reactive T-cell clones are eliminated by negative selection in the process of T-cell maturation, and the remaining self-reactive T-cell clones are rendered anergic in the peripheral or germinal center. However, self-reactive T-cell clones sometimes emerge and result in autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [1,2].

Many reports exist on the genetic association of HLA with diseases, especially autoimmune diseases [3]. In autoimmune diseases, elevated proinflammatory cytokine levels in peripheral blood are often observed [4]. For example, the tumor necrosis factor α (TNF-α) concentration in plasma in RA patients is increased [5], and successful outcomes in RA treatment have been achieved using anti-TNF-α or TNF-α pathway antibodies, including anti-interleukin (IL)-6 antibody [6,7]. On the other hand, it has recently been elucidated that obesity-induced chronic inflammation is involved in various diseases [8–10]. TNF-α is overexpressed in the adipose tissue of the rodent model of obesity [11] and is a candidate mediator of insulin resistance in obesity. In addition, TNF-α-deficient obese mice are protected from obesity-induced insulin resistance [12]. In obesity, the IL-6 level is also elevated and IL-6 promotes insulin resistance [13,14]. High-density lipoprotein cholesterol (HDL cholesterol) is also involved in inflammation [15]. In metabolic syndromes such as type 2 diabetes and obesity, the plasma level of HDL cholesterol is significantly decreased and the plasma levels of proinflammatory cytokines are increased inversely [16]. Similarly, an increase in TNF-α, IL-1, and IL-6 levels and a decrease in HDL cholesterol level were observed in SLE and RA patients. An increase of low-density lipoprotein cholesterol (LDL cholesterol) and triglyceride was also observed in SLE and RA patients [17–19].

Given the above findings, particularly on the associations between HLA and autoimmune diseases in which elevated proinflammatory cytokine levels are often observed between obesity-induced chronic inflammation and plasma levels of biochemical

* Corresponding author.
E-mail address: hinoko@is.icc.u-tokai.ac.jp (H. Inoko).

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traits and between autoimmune diseases and plasma levels of biochemical traits, we became interested in investigating the association of HLA alleles with biochemical traits such as fasting blood glucose, total cholesterol, and HDL cholesterol as intermediate phenotypes of various diseases including diabetes and cardiovascular diseases. Therefore, we conducted association analyses among 6 *HLA* loci (*HLA-A, -B, -C, -DRB1, -DQB1,* and *-DPB1*) and 7 biochemical traits: fasting blood glucose, HbA1c, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, and uric acid.

2. Subjects and methods

2.1. Subjects

A total of 1,616 individuals who visited the Health Evaluation and Promotion Center of Tokai University Hospital were included in this study. Blood sampling was performed in a 12-hour fasting state. All subjects gave their written informed consent to participate. Approval from the ethical committee of Tokai University School of Medicine was also obtained.

2.2. HLA typing

DNA samples extracted from peripheral blood using the standard method were used for *HLA* typing. We analyzed 6 *HLA* loci (HLA-A, -B, -C, *-DRB1, -DQB1,* and *-DPB1*) using the Luminex assay system and HLA typing kits (WAKFlow HLA typing kits, Wakunaga, Osaka, Japan, or LABType SSO, One Lambda, Canoga Park, CA). Although we typed *HLA-DRB1* in all of the 1,616 subjects, we typed 5 *HLA* loci, *HLA-A, HLA-B, HLA-C, HLA-DQB1,* and *HLA-DPB1,* for 1,047 of the 1,616 subjects.

The observed *HLA* alleles and their frequencies in this study were compared with the allele frequencies reported by the Central Bone Marrow Data Center, Japanese Red Cross Society (http://www.bmdc.jrc.or.jp/stat.html) for *HLA-A, HLA-B, HLA-C,* and *HLA-DRB1* and those reported by the HLA Laboratory (http://www.hla.or.jp/) for *HLA-DQB1* and *HLA-DPB1*. The observed allele frequencies exhibited good concordance with the reported allele frequencies. *HLA* alleles that had more than 1% frequencies were used for the following analyses. The allele numbers used for statistical analyses and the observed allele numbers were as follows (analyzed/observed): 11/23 for *HLA-A,* 19/40 for *HLA-B,* 11/19 for *HLA-C,* 19/31 for *HLA-DRB1,* 11/14 for *HLA-DQB1,* and 9/17 for *HLA-DPB1*. The allele frequencies observed in this study are presented in Supplementary Tables 1–6.

2.3. Statistical analyses

We used Statcel 2 software (OMS, Saitama, Japan) to test the normality of each biochemical trait distribution. Because none of the 7 biochemical traits, except for total cholesterol, was normally distributed, Mann–Whitney *U* tests and Fisher's exact tests were conducted in this study using the R software. We divided the subjects into 2 groups, 1 group with a specific HLA allele and the other without, and used the Mann–Whitney *U* test to analyze the biochemical traits of the 2 groups. Fisher's exact tests were conducted between the groups within reference values and the abnormal-value group. The following criteria were used for grouping: <110 (within reference value) and ≥126 mg/dL (abnormal value) for fasting plasma glucose, <5.6% and ≥6.0% for HbA1c, <220 and ≥240 mg/dL for total cholesterol, <140 and ≥160 mg/dL for LDL cholesterol, ≥40 and <40 mg/dL for HDL cholesterol, <150 and ≥200 mg/dL for triglyceride, <6.0 and ≥7.0 mg/dL for female uric acid, and <7.0 and ≥8.0 mg/dL for male uric acid. p_c was calculated by multiplying the *p* value by the number of *HLA* analyzed, namely, the number of *HLA* alleles with an allele frequency of more than 1%. Statistical power analysis was performed using G*Power [20]. *HLA* haplotypes were estimated using PHASE version 2.1 [21].

3. Results

The characteristics of the subjects are summarized in Table 1. Compared with the statistical data issued by the Japanese government (http://www.mext.go.jp/b_menu/toukei/001/index22.htm), there were no significant differences in the height and weight among the subjects. Some biochemical traits demonstrated significant differences between males and females (Table 1), as indicated in the previous report based on analysis of the health examination data obtained from 700,000 Japanese individuals [22]. Therefore, we performed statistical analyses between the *HLA* alleles and biochemical traits separately by gender as well as for the overall subjects.

We analyzed the association between *HLA* alleles and biochemical traits using the Mann–Whitney *U* test. The results are presented in Table 2. The female subjects with *DPB1*03:01* demonstrated significant differences from the female subjects without *DPB1*03:01* on HbA1c (p_c = 0.000598) after Bonferroni correction. The female subjects with *DPB1*0301* also demonstrated significant differences (p_c = 0.0370) in fasting plasma glucose level, although the statistical power was low (0.21). Similarly, *DRB1*14:03* on the total cholesterol concentration (p_c = 0.0287) and *C*14:03* on the triglyceride concentration (p_c = 0.0205) demonstrated significant differences in female subjects. Although LDL cholesterol is a major component of total cholesterol and *DRB1*14:03* exhibited a tendency (p = 0.0160) to be associated with LDL cholesterol, it was not significant after Bonferroni correction. In male subjects, *A*26:02* (p_c = 0.0399) and *C*14:02* (p_c = 0.0452) on fasting plasma glucose level demonstrated significant differences. The *HLA* alleles that demonstrated significant differences in female subjects demonstrated no significant differences in male subjects. In the overall subjects, *C*14:03* on triglyceride concentration (p_c = 0.0067) and *DPB1*03:01* on the HbA1c (p_c = 0.0432) demonstrated significant differences. Although other *HLA* alleles also demonstrated significant differences resulting from strong linkage disequilibrium (LD) between *HLA* loci, only *HLA* alleles that demonstrated the lowest *p* value on the haplotypes are listed in Table 2. Estimated haplotypes and their frequencies using PHASE demonstrated good concordance with those previously reported (data not shown) [23].

Fisher's exact tests were performed using the group within reference biochemical values and the group with abnormal values. The results are shown in Table 3. Total cholesterol and LDL cholesterol exhibited significant differences in the female subjects with *DRB1*14:03* (p_c = 0.0061, odds ratio [OR] = 4.32, 95% confidence interval [95% CI] = 1.83–10.36) and *DPB1*02:01* (p_c = 0.0389, OR = 0.41, 95% CI = 0.19–0.79) after Bonferroni correction, respectively. The male subjects demonstrated no significant differences. The *DRB1*14:03* alleles in female subjects exhibited significant *p* values in both the Mann–Whitney *U* test and the Fisher exact test.

We could not detect any significant association or differences in HDL cholesterol and uric acid after Bonferroni correction. Although the C-reactive protein (CRP) level is an inflammatory biomarker, we could not detect any significant association with body mass index (BMI) and biochemical traits either because of low effect size. When the means and standard deviation of the CRP level were calculated by substituting <0.09 by 0.08, the effect size was 0.197 for *DRB1*1403,* which demonstrated significant differences in both the Mann–Whitney *U* test and the Fisher exact test.

4. Discussion

We detected several associations of *HLA* alleles with biochemical traits using the Mann–Whitney *U* test and Fisher's exact test. Total cholesterol consists of LDL cholesterol, HDL cholesterol, and triglyceride-rich lipoprotein cholesterol; the major component is LDL cholesterol. *HLA-DRB1*14:03* is associated with the total cho-

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Table 1
Subject characteristics and comparison of biochemical values

	Female	Male	Total	p value
N	772	844	1,616	
Age (years)	56.3 ± 10.1	57.9 ± 10.2	57.1 ± 10.2	
Height (cm)	155.8 ± 5.6	168.5 ± 6.0	162.4 ± 8.6	
Weight (kg)	53.0 ± 8.0	66.6 ± 9.6	60.1 ± 11.2	
BMI (kg/m^2)	21.8 ± 3.1	23.4 ± 3.0	22.7 ± 3.1	
Fasting plasma glucose (mg/dL)	97.1 ± 13.6[a]	103.2 ± 15.9	100.3 ± 15.1	1.98E-16
HbA1c (%)	5.08 ± 0.47	5.12 ± 0.61	5.10 ± 0.55	0.184
Total cholesterol (mg/dL)	210 ± 31.0	203 ± 30.3	207 ± 30.9	1.44E-07
LDL cholesterol (mg/dL)	125 ± 28.6	125 ± 28.7	125 ± 28.6	0.849
HDL cholesterol (mg/dL)	74.4 ± 18.3	62.1 ± 16.9	68.0 ± 18.6	6.6E-42
Triglyceride (mg/dL)	91.3 ± 49.9	124 ± 73.3	108 ± 65.3	2.54E-25
Uric acid (mg/dL)	4.59 ± 1.00	5.98 ± 1.15	5.31 ± 1.28	4E-125
CRP (mg/dL)[b]	0.125 ± 0.267	0.142 ± 0.335	0.134 ± 0.304	0.274

Student's *t* tests were used for total cholesterol and LDL cholesterol that demonstrated equality of variance in the *F* test, and Welch's *t* tests were used for the other biochemical traits that demonstrated unequal variance.
[a]Values are means ± SD.
[b]Means and standard deviation were calculated by substituting <0.09 by 0.08.

lesterol concentration in female subjects when evaluated by both Mann–Whitney *U* test and Fisher's exact test. However, we could not detect an association of *HLA-DRB1*14:03* with LDL cholesterol using both tests ($p_c = 0.738$ for the Mann–Whitney U test and $p_c = 0.238$ for Fisher's exact test) because of low statistical power. *DPB1*02:01* is protective to the concentration of LDL cholesterol when evaluated by Fisher's exact test and *HLA-C*14:03* is associated with triglyceride concentration in female subjects when evaluated by Mann–Whitney *U* test.

Many genome-wide association studies (GWAS) on dyslipidemia have recently been conducted, and numerous related genes were reported [24–28]. A few reports indicate that genes located within or near the *HLA* loci are related to dyslipidemia, although the associations of *DRB1*14:03* (allele frequency 1.8%) with total cholesterol and *C*14:03* (allele frequency 8.6%) with triglyceride were observed by the Mann–Whitney *U* test in this study. It is likely that *HLA* alleles with relatively high allele frequency (more than 10%) and high OR could be detected by GWAS, whereas those with low allele frequency (less than 10%) could not. In this study, *DPB1*02:01* is protective to the concentration of LDL cholesterol when evaluated by Fisher's exact test. A single nucleotide polymorphism (SNP), rs2254287, which is located near the *B3GALT4* downstream of *DPB1*, was reported to be associated with LDL cholesterol by GWAS [29]. It remains unclear whether the rs2254287 association is the result of LD with *DPB1*02:01* because the HapMap database does not contain DPB1 allele data. This must be elucidated in the future.

We reported that *DPB1*03:01* is associated with HbA1c and fasting plasma glucose in female subjects and that *A*26:02* and *C*14:02* are associated with fasting plasma glucose in male subjects. The 2 alleles of *A*26:02* and *C*14:02* are not in LD to each other because they were not assigned to the same haplotype by PHASE analysis. Although a striking difference was observed in the average BMI of type 2 diabetes patients among different human populations, type 2 diabetes is related to obesity, and DR4 confers the protective effect to type 2 diabetes in Caucasians [30]. We could not detect any association of *DRB1*04* with fasting plasma glucose level.

Bromodomain-containing protein 2 (BRD2) is a putative nuclear transcriptional regulator and a member of a family of genes that are expressed during development (OMIM 601540). The *BRD2* locus is located between *HLA-DMA* and *HLA-DMO*, 91 kb upstream of the *DPB1* locus in the HLA class II region. Interestingly, it has been reported that *Brd2* disruption in mice caused severe obesity without type 2 diabetes [31]. In this paper, the participation of *Brd2* in TNF-α signaling to induce insulin resistance *in vitro* was indicated, and lowered levels of Brd2 reduced inflammatory signal transduction *in vivo*. In the present study, *DPB1*03:01* was significantly associated with HbA1c by the Mann–Whitney *U* test. Although the minor allele frequencies of all SNPs in the *BRD2* locus so far reported are low in the Japanese population (dbSNP), *DPB1*03:01* may be in linkage disequilibrium with the particular SNP(s) in the *BRD2* locus that affects the expression level of the *DRB2* gene. The LD block of the haplotype with *DPB1*03:01* should be studied to clarify whether there is an association with

Table 2
Associations between HLA alleles and biochemical traits in the Mann–Whitney *U* test

	Female			Male			Total		
	p	p_c	Power	*p*	p_c	Power	*p*	p_c	Power
Fasting plasma glucose									
A*26:02	0.2865	1	0.24	0.0036	0.0399	0.78	0.0079	0.0874	0.75
C*14:02	0.8608	1	0.10	0.0041	**0.0452**	**0.90**	0.0302	0.3318	0.82
DPB1*03:01	0.0041	0.0370	0.21	0.6299	1	0.20	0.0170	0.1526	0.37
HbA1c									
DPB1*03:01	0.0000665	**0.000598**	**0.82**	0.7547	1	0.22	0.0048	0.0432	0.68
Total cholesterol									
DRB1*14:03	0.0015	**0.0287**	**0.83**	0.3041	1	0.15	0.0028	0.0541	0.81
LDL cholesterol									
DPB1*02:01	0.0160	0.1440	0.80	0.8864	1	0.05	0.0742	0.6678	0.50
Triglyceride									
C*14:03	0.0019	0.0205	0.72	0.0504	0.5542	0.53	0.000605	**0.0067**	**0.85**

p_c was calculated by multiplication of the allele number that used statistical analysis (allele frequency >1.0%) by the *p* value. When p_c is more than 1, p_c is expressed as 1. Only the alleles that demonstrated the smallest p_c value on the haplotype with $p_c < 0.05$ are shown. The p_c values that are less than 0.05 with more than 0.8 of statistical power are shown in boldface.

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Table 3
Associations between HLA alleles and biochemical traits on Fisher's exact test

	Female	Male	Total
Total cholesterol ≥240 versus <220 (mg/dL)			
DRB1*14:03			
p	0.000323	0.5456	0.0011
p_c	0.0061	1	0.0206
Power	0.93	0.084	0.87
OR	4.32	1.32	2.77
95% CI	1.83–10.36	0.33–4.01	1.45–5.15
LDL cholesterol: ≥160 versus <140 (mg/dL)			
DPB1*02:01			
p	0.0043	1	0.0544
p_c	0.0389	1	0.4897
Power	0.84	0.045	0.51
OR	0.41	0.97	0.69
95% CI	0.19–0.79	0.58–1.56	0.46–1.00

p_c was calculated by multiplication of the allele number that used statistical analysis (allele frequency >1.0%) by the *p* value. Only the alleles the demonstrated the smallest p_c value on the haplotype with $p_c < 0.05$ are shown.

particular SNP(s) in the *BRD2* locus by resequencing study of this gene region.

Although many GWAS were conducted on type 2 diabetes [32], association of *HLA* with type 2 diabetes was not reported. The type 2 diabetes susceptibility genes on the 6p arm around the HLA region, which was replicated in several populations by GWAS, was mapped only around the *CDKAL1* locus. However, because the gene is about 9 Mb apart from the *HLA-A* locus, it is unlikely that *CDKAL1* lies in LD with *HLA-A*. The observed allele frequencies of *DPB1*03:01*, *A*26:02*, and *C*14:02* in this study are 4.4, 1.8, and 7.4%, respectively. The relatively low allele frequencies may be one reason why the association cannot be detected in GWAS.

A close correlation of soluble HLA-G (sHLA-G) antigen with insulin resistance was reported [33]; sHLA-G was more frequently detected in type 2 diabetes than in the healthy population. The 14-bp insertion/deletion polymorphism in exon 8 of the *HLA-G* gene was significantly associated with sHLA-G expression and the plasma sHLA-G level with the +14 bp/+14 bp genotype was remarkably lower than that with the +14 bp/−14 bp and −14 bp/−14 bp genotypes [34]. In addition, strong LD was reported between the sHLA-G14 bp deletion and *HLA-A*02*, *-A*03*, and *-A*24* [35]. Taken together with these reports, it is likely that *HLA-A*02*, *-A*03*, and *-A*24* are associated with a higher fasting plasma glucose level. However, we could not detect any association between *HLA-A*02*, *-A*03*, and *-A*24* and fasting plasma glucose level.

The biochemical traits studied here are intermediate phenotypes between healthy and disease states. Therefore, many other factors including environmental factors may be involved in disease onset. In this study, not all *HLA* alleles that demonstrated significant associations with biochemical traits in the Mann–Whitney *U* test were significant by Fisher's exact tests. This may reflect that individuals demonstrating a relatively high value of biochemical traits within reference values need other genetic and environmental factors for shifting to the abnormal-value group. Although this study demonstrated significant associations between particular *HLA* alleles and biochemical traits using more than 1,000 cases for the first time in the Japanese population, further analyses are required to confirm the associations.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.humimm.2011.03.011.

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