**HLA-A*33-DR3 and A*33-DR9 haplotypes enhance the risk of type 1 diabetes in Han Chinese**

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**ABSTRACT**

**Aims/Introduction:** To investigate the typing for human leukocyte antigen (HLA) class I in Chinese patients with type 1 diabetes as a complement screening for HLA class II.

**Materials and Methods:** A total of 212 type 1 diabetic patients and 200 healthy controls were enrolled. The genetic polymorphisms of HLA class I and II were examined with a high-resolution polymerase chain reaction sequence-based typing method.

**Results:** The haplotype, A*33:03-B*58:01-C*03:02(A33), was associated with type 1 diabetes (P = 1.0 x 10^-4, odds ratio 3.2 [1.738–5.843]). The A33-DR3 and A33-DR9 haplotypes significantly enhanced the risk of type 1 diabetes (A33-DR3, odds ratio 5.1 [2.40–10.78], P = 4.0 x 10^-6; A33-DR9, odds ratio 13.0 [1.69–100.32], P = 0.004). In type 1 diabetic patients, compared with A33-DR3-negative carriers, A33-DR3-positive carriers had significantly lower percentages of CD3^CD4^ T cells (425 ± 7.72 vs 370 ± 8.35%, P = 0.023), higher percentages of CD3^CD8^ T cells (27.4 ± 7.09 vs 32.8 ± 5.98%, P = 0.005) and T-cell receptor α/β T cells (70.0 ± 7.00 vs 73.6 ± 6.25%, P = 0.031), and lower CD4/CD8 ratios (1.71 ± 0.75 vs 1.16 ± 0.35, P = 0.003).

**Conclusions:** It is the first time that the haplotypes A33-DR3 and A33-DR9 were found with an enhanced predisposition to type 1 diabetes in Han Chinese. A33-DR3 was associated with a reduction in the helper-to-cytotoxic cell ratio and preferential increase of T-cell receptor α/β T cell. The typing for HLA class I and its immunogenetic effects are important for more accurate HLA class II haplotype risk prediction and etiology research in type 1 diabetic patients.

**INTRODUCTION**

Type 1 diabetes is an autoimmune disease characterized by immune-mediated destruction of pancreatic β-cells, resulting in permanent β-cell loss and insulin deficiency. It is widely recognized that the major component of human leukocyte antigen (HLA) susceptibility to type 1 diabetes involves the DRB1, DQA1 and DQB1 genes. Recent studies have suggested that genes in the HLA region other than DR and DQ also contribute to type 1 diabetes susceptibility.

The association of HLA class I polymorphisms with the age at onset of type 1 diabetes in Caucasian populations has been previously reported. HLA class I molecules, given their role in target-cell recognition by CD8^+ cytotoxic T lymphocytes, play a role in the ongoing immune response and, therefore, could affect the rate of pancreatic β-cell destruction. This hypothesis might help explain the observed associations of HLA class I with type 1 diabetes, particularly the association of HLA class I with lymphocyte subpopulation. In addition, it has...
recently been observed that innate immune cell cross-talk also occurs in the pancreas of young NOD mice and leads to the initiation of type 1 diabetes, with the interaction of immunoglobulin G (IgG)-producing B-1a cells, neutrophils and interferon-α-producing pDCs.

With the requirement of secondary type 1 diabetes prevention trials and the selection of participants with impending diabetes, and the fact that thus far, scarce research is available regarding the association of T lymphocyte subpopulations and innate immunity with high-risk HLA class I genes in patients with type 1 diabetes at the disease onset, we investigated whether typing for HLA-A, HLA-B and HLA-C might complement screening for HLA-DRB1, DQB1 and DQA1 in Chinese patients in Eastern China, and sought to characterize the clinical and immunological features of HLA-type youth with new-onset type 1 diabetes.

MATERIALS AND METHODS

Study Cohort

A total of 212 patients with new-onset type 1 diabetes included in this dataset were identified and examined at the Department of Endocrine and Metabolic diseases, Ruijin Hospital Affiliated to Shanghai Jiao-Tong University School of Medicine, Shanghai, China, from April 2006 to October 2012. The average age at diagnosis was 20.2 ± 5.5 years, and the average duration of diabetes was 10 ± 6.4 weeks with 87 men and 125 women. A total of 200 healthy volunteers were included as controls, including 117 men and 83 women, with an average age of 23.6 ± 4.2 years, and had no family history of diabetes or overt autoimmune diseases and any chronic diseases. All the cases and controls were Han Chinese and from the East Chinese population belonging to the East Chinese states of Shanghai, Jiangsu and Zhejiang. Individuals with type 1 diabetes or not diagnosed according to the diagnosis criteria of the World Health Organization and American Diabetes Association.Agreement with the Declaration of Helsinki as revised in 2000. The serum levels of anti-glutamic acid decarboxylase antibodies (IgG) were measured by a radioimmunoassay using a commercially available kit (Roche Diagnostics, Penzberg, Germany). The lower limit of detection was 0.1 ng/mL, and undetected values were reported as 0.1 ng/mL. The serum levels of anti-glutamic acid decarboxylase antibodies were also measured by a radioimmunoassay using a commercial

Genotyping Methods

HLA genotyping detection was carried out on 212 patients with type 1 diabetes and 200 healthy controls, with six loci, including A, B, C, DRB1, DQB1 and DQA1, with a polymerase chain reaction sequence-based typing system (Shanghai Tissuebank Biotechnology Co., Ltd., Shanghai, China). Genomic deoxyribonucleic acid was isolated from whole blood using the QiaAMP® DNA mini kits (Qiagen GmbH, Hilden, Germany), and used to amplify HLA class I and class II genes by polymerase chain reaction with primers listed in Table S1. Amplicons were purified using ExoSAP protocols (Exonuclease I and Alkaline Phosphatase kits; Takara, Dalian, China) and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Ready kits (Applied Biosystems, Foster City, CA, USA) with the specific sequencing primers (Table S2). Sequences were analyzed using the ABI 3730xl genetic analyzer (Applied Biosystems). The sequences were then genotyped using uTYPE3.0 software and database 3.7 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Class I HLA alleles were sequenced for exons 2, 3 and 4 in both directions, and class II HLA alleles were sequenced for exon 2 and 3 in both directions. To determine the ambiguity, allele-specific sequencing was carried out using a set of group-specific primers (HLA-SBT kit; CATB, Shanghai, China). The deoxyribonucleic acid database of the same sequences of all known alleles were thereby compared to obtain accurate allele coding.

Flow Cytometry

Blood collection was carried out in 212 patients with type 1 diabetes and 10 healthy controls. The measurements of forward and side scatter were combined with CD45 to identify lymphocytes and exclude monocytes. The absolute subpopulations of lymphocyte numbers were calculated based on the total lymphocyte count and the percentage of subpopulations of lymphocyte cells, as identified by flow cytometry using the EPICS XL (Beckman Coulter, Brea, CA, USA) and Diva software (BD Biosciences, Franklin Lakes, NJ, USA). The fraction of lymphocyte cell subsets was determined by multiple-colors fluorescence activated cell sorter analysis using appropriate surface markers: anti-CD3-FITC (Clone UCHT1), anti-CD4-PE-Cy7 (Clone 13B8.2), anti-CD8-APC-H7 (Clone B9.11), anti-CD45-PerCP-Cy5.5 (Clone J.33), anti-CD45RA-FITC (Clone ALB11), anti-C62L-PE (Clone DREG56), anti-CD19-APC (Clone J3.119) anti-CD16-PE (Clone 3GB), anti-CD56-PE (Clone N901), anti-TCR PAN α/β-FITC (TCR 1, Clone IP26A) and anti-TCR PAN γδ-PE (TCR 2, Clone IMMU510; Beckman Coulter).

Laboratory Assessment of Diabetes Status

The serum C-peptide levels were measured by a radioimmunoassay using a commercially available kit (Roche Diagnostics, Penzberg, Germany). The lower limit of detection was 0.1 ng/mL, and undetected values were reported as 0.1 ng/mL. The serum levels of anti-glutamic acid decarboxylase antibodies were also measured by a radioimmunoassay using a commercial
kit (RSR Limited, Cardiff, UK), and the results were considered positive if the values were >7.5 units/mL. Glycated hemoglobin was measured by high-pressure liquid chromatography. The serum levels of hs-CRP were measured using the latex enhanced immunoturbidimetric method. Both IgG and IFN-γ were measured by enzyme-linked immunosorbent assay (Gentaur, Kampenhout, Belgium) and flow cytometry (Beckman, High Wycombe, UK), respectively.

Statistical Analysis

The Hardy–Weinberg test of HLA alleles and their linkage disequilibrium test results were analyzed using Arlequin 3.11 software (Institute of Ecology and Evolution, University of Bern, Bern, Switzerland). Statistical analysis was carried out with SPSS (Institute of Ecology and Evolution, University of Bern, Switzerland). Statistical analysis was carried out with SPSS 17.0 (SPSS, Armonk, NY, USA). The results are shown as the mean ± standard deviation or as median (range), or otherwise documented as positive cases, constituent ratio or ratio. The difference between classified variables was tested using the χ²-test or Fisher’s exact test if the expected number of participants in any cell was less than five. ANOVA analyses were carried out among the groups with a model that included the baseline values of the age of onset and duration of diabetes as covariates. C-peptide, anti-glutamic acid decarboxylase antibodies and hs-CRP were studied on the logarithmic scale. All hypothesis testing was two-tailed. We made the Bonferroni correction for the P-values by the number of alleles with substantial frequencies (≥5%) in six loci including HLA-A, B, C, DRB1, DQB1 and DQA1 respectively. Statistical significance was defined as P < 0.05.

RESULTS

HLA Alleles and Haplotypes

The association of HLA alleles and haplotypes with type 1 diabetes is shown in Tables 1, S3 and S4. The frequencies of A*24:02, B*58:01, C*03:02 and A*33:03-B*58:01-C*03:02, DRB1*03:01-DQA1*05:01-DQB1*02:01(DR3), DRB1*04:02-XX-DQA1*03:01-DQB1*03:02(04XX: 04:01,04:04,04,05) (DR4) and DRB1*09:01-DQA1*03:02-DQB1*03:03(DR9) haplotypes were significantly higher in the patients with type 1 diabetes than the healthy controls (all P < 0.05).

The differences in allele frequencies between controls and cases for these alleles could be exclusively due to the strong linkage disequilibrium between the HLA class I and class II loci. To address this issue, we computed the normalized linkage disequilibrium in the type 1 diabetic patients (Table 2). The strongest linkage disequilibrium observed in both samples was between the well-known high-risk haplotypes, DRB1*03:01-DQB1*02:01 and DRB1*09:01-DQB1*03:03, respectively. The other apparently predisposing allele, A*24:02, showed no significant linkage disequilibrium to any particular DR-DQ haplotype. Of the apparently predisposing alleles, A*33:03 showed a very strong linkage disequilibrium with B*58:01, which explains why A*33:03 often appeared with B*58:01 among patients, whereas both these two alleles showed no significant linkage disequilibrium with the high-risk haplotypes DR3, DR4 and DR9 (Tables S5–10). Accordingly, primary association data for HLA region markers remained significantly associated with type 1 diabetes after computation of linkage disequilibrium.

We then assessed the risk of both the A33 haplotype and A*24:02 allele with these stratified HLA-DR-DQ haplotypes compared with the results presented earlier, which were not stratified by HLA-DR-DQ haplotype. Haplotype A33 was associated with case chromosomes on DR3 (P = 4.0 × 10⁻⁶, odds ratio 5.09) and is also associated with DR9 haplotypes (P = 0.004, odds ratio 13.00; Table 3), with the exception of DR4, where there was no increased risk associated with the A33 haplotype (P = 0.499).

Clinical Characteristics in Patients with High-risk A33-DR3 and A33-DR9 Haplotypes

To explore whether there were cooperative effects between the A33 and high-risk DR3 or DR9 haplotypes, we assessed the correlation between clinical characteristics and A33 haplotype on DR3 or DR9. The participants were divided into two groups according to different high-risk haplotypes. Haplotypes A33-DR3 and A33-DR9 were detected in 41 and 13 out of 212 patients with type 1 diabetes, respectively (19.3% and 6.1%). There were 126 and 92 participants in the A33-DR3-negative and A33-DR9-negative subgroups, respectively (39.4% and 43.4%). A33-DR3-positive carriers were referred to as patients with both haplotype A33 and haplotype DR3, whereas A33-DR3-negative carriers were identified as participants without either haplotype A33 or haplotype DR3. The identification of A33-DR9-positive and A33-DR9-negative subgroups was the same as that of the A33-DR3 subgroups. Clinical features, such as the age at onset, duration of diabetes, frequency of presence with diabetic ketoacidosis history and relative with type 1 diabetes, levels of fasting C-peptide, peak value of C-peptide during a 75-g oral glucose tolerance test, and area under C-peptide release curve during an oral glucose tolerance test, showed no significant differences between A33-DR3-positive carriers and A33-DR3-negative carriers (Table S11). For the A33-DR9-positive and A33-DR9-negative subgroups, there were also no significant differences in any clinical characteristics.

Immunological Features in Type 1 Diabetic Patients Consistent with High-risk A33-DR3 and A33-DR9 Haplotypes

Haplotype A33-DR3 was associated with the imbalance of T lymphocyte subpopulations at the onset of type 1 diabetes (Figure 1), and an example of the gating strategy and fluorescence activated cell sorter plots is shown in Figure 2. Compared with A33-DR3-negative carriers, A33-DR3-positive carriers had a significantly lower percentage of CD3⁺CD4⁺ T cells (42.5 ± 7.72 vs 37.0 ± 8.35%, P = 0.023), higher percentage of CD3⁺CD8⁺ T cells (27.4 ± 7.09 vs 32.8 ± 5.98%, P = 0.005) and lower level of ratio of CD4/CD8 (1.71 ± 0.75 vs 1.16 ± 0.35, P = 0.003). In addition, A33-DR3-positive carriers had a significantly higher percentage of T-cell receptor (TCR)
### Table 1 | Comparison of human leukocyte antigen gene frequencies between type 1 diabetic patients and healthy controls

| Gene                  | Type 1 diabetic patients (n = 212) | Healthy controls (n = 200) | P     | Pc    | OR (95% CI) |
|-----------------------|-----------------------------------|---------------------------|-------|-------|-------------|
| Alleles               |                                   |                           |       |       |             |
| HLA-A                 |                                   |                           |       |       |             |
| *A*24:02              | 85 (40.1)                         | 44 (22.0)                 | 7.6 × 10⁻⁵ | 6.86 × 10⁻⁴ | 2.373 | 1.539–3.658 |
| *A*33:03              | 55 (25.9)                         | 36 (18.0)                 | 0.052 | 0.468 | 1.596 | 0.994–2.563 |
| HLA-B                 |                                   |                           |       |       |             |
| *B*58:01              | 62 (29.2)                         | 21 (10.5)                 | 2.1 × 10⁻⁶ | 2.7 × 10⁻⁵ | 3.523 | 2.052–6.048 |
| HLA-C                 |                                   |                           |       |       |             |
| *C*03:02              | 60 (28.3)                         | 21 (10.5)                 | 5.5 × 10⁻⁶ | 5.0 × 10⁻⁵ | 3.365 | 1.957–5.785 |
| Haplotype             |                                   |                           |       |       |             |
| A-B-C                 |                                   |                           |       |       |             |
| *A*33:03-*B*58:01-*C*03:02 | 46 (21.7)                         | 16 (8.0)                  | 1.0 × 10⁻⁴ | – | 3.187 | 1.738–5.843 |
| DRB1-DQB1-DQA1        |                                   |                           |       |       |             |
| DR3                   | 81 (38.2)                         | 26 (13.0)                 | 5.5 × 10⁻⁹ | – | 4.138 | 2.518–6.799 |
| DR4                   | 31 (17.1)                         | 3 (1.5)                   | 3.2 × 10⁻⁵ | – | 11.247 | 3.380–37.420 |
| DR9                   | 87 (41.0)                         | 42 (21.0)                 | 1.2 × 10⁻⁵ | – | 2.618 | 1.692–4.052 |

Data are expressed as n (%). χ² or Fisher’s exact test. DR3 = DRB1*03:01-DQA1*05:01-DQB1*02:01, DR4 = DRB1*04:XX-DQA1*03:02-DQB1*03:02(04:XX:04:01, 04:04, 04:05), DR9 = DRB1*09:01-DQA1*03:02-DQB1*03:03. – Not available; CI, confidence interval; HLA, human leukocyte antigen; OR, odds ratio; Pc, corrected P-values.

### Table 2 | Normalized linkage disequilibrium between human leukocyte antigen class I and human leukocyte antigen class II alleles (n = 212)

| Haplotype | D'   | r²   | χ²  | P-value |
|-----------|------|------|-----|---------|
| HLA-A     |      |      |     |         |
| 33:03     | 58:01 | 0.76 | 0.47 | 171.06  | <0.001  |
| DRB1      | DQB1 | 0.90 | 0.80 | 288.70  | <0.001  |
| 03:01     | 02:01 | 0.72 | 0.52 | 188.98  | <0.001  |
| 09:01     | 03:03 | 0.80 | 0.39 | 140.84  | <0.001  |

Only haplotypes that showed significant linkage disequilibrium are listed. HLA, human leukocyte antigen.

α/β(TCR1⁺) T cells than the A33-DR3-negative carriers (73.6 ± 6.25 vs 70.0 ± 7.00%, P = 0.031). However, the percentages of CD45RA⁺CD62L⁺ lymphocytes, neutrophils, natural killer (CD16⁺CD56⁺) cells and the IgG-producing B (CD19⁺) cells were not significantly different between the two subgroups. None of the serum protein levels measured, such as glutamic acid decarboxylase antibodies, hs-CRP, IgG and IFN-γ, appeared to be associated with A33-DR3. No significant differences were found in any immunological features between A33-DR9-positive carriers and A33-DR9-negative patients, except for CD3⁺CD4⁺ T cells and CD4⁺CD8⁺, respectively (34.8 ± 13.71 vs 44.3 ± 8.48%, P = 0.022; 1.07 ± 0.46 vs 1.76 ± 0.72, P = 0.042). To evaluate whether the observed associations of an altered phenotype in lymphocytes is associated with the A33-DR3 haplotype or with type 1 diabetes, we tested the α/β T lymphocyte subpopulations in 10 healthy participants to evaluate controls by flow cytometry. The data is organized as Figures 1, S1 and S2. Although there were no significant differences in any immunological features between CD3⁺CD4⁺ T cells and CD3⁺CD8⁺ T cells, A33-DR3-positive carriers had the lowest ratio of CD4/CD8. Compared with healthy controls, A33-DR3-positive carriers and A33-DR3-negative carriers had a significantly higher percentage of TCRα/β T cells, respectively (58.7 ± 9.12 vs 73.6 ± 6.25%, P = 2.6 × 10⁻⁵; 58.7 ± 9.12 vs 69.7 ± 7.00%, P = 9.5 × 10⁻⁷). Compared with A33-negative carriers, A33-positive carriers also had a significantly lower level of ratio of CD4/CD8 (1.75 ± 0.68 vs 1.16 ± 0.35, 2.618 ± 1.682 vs 1.16 ± 0.35, 5.5 × 10⁻⁶ vs 2.7 × 10⁻⁵).

### Table 3 | Analysis of case and control chromosomes for the risk associated with the haplotype A*33:03-*B*58:01-*C*03:02, stratified by HLA-DR-DQ haplotype

| Haplotype | Type 1 diabetes patients (n = 212) | Healthy control (n = 200) | OR (95% CI) | P    |
|-----------|-----------------------------------|---------------------------|-------------|------|
|           | With A33*                           | With A33                   |             |      |
| DR3       | 41                                 | 9                         | 5.09 (2.40–10.78) | 4.0 × 10⁻⁶ |
| DR4       | 2                                  | 0                         | NA          | 0.499 |
| DR9       | 13                                 | 1                         | 13.00 (1.69–100.32) | 0.004 |

*A33 = A*33:03-*B*58:01-*C*03:02. χ² or Fisher’s exact test. CI, confidence interval; NA, not available; OR, odds ratio.
P = 2.3 × 10^{-6}). No significant differences were found in CD4/CD8 ratio between DR3-positive carriers and DR3-negative patients.

**DISCUSSION**

Several studies have suggested that extended or ancestral major histocompatibility complex haplotypes provide an even greater risk to develop autoimmunity compared with individual alleles of any single gene\(^9,10\). Thus, we mainly focused on the susceptibility of haplotypes for HLA class I/II in the present study.

In our study, we defined two sets of HLA-A33\(^*\) haplotypes that provide maximum risk of type 1 diabetes development in Chinese: the A33-DR3 haplotype and A33-DR9 haplotype. According to published literature, A\(^*\)33:03-B\(^*\)58:01-C\(^*\)03:02-DRB1\(^*\)03:01 is reported as a relatively common haplotype in the Han Chinese population\(^11-13\). As there were no linkage disequilibrium between the haplotype A33 and DR3, the positive association of A33-DR3 with type 1 diabetes might be the accumulation of specific HLA class I and class II. In the North Indian population, A2-B8-DR3 contributed the maximum risk (relative risk = 48.7) for type 1 diabetes, followed by A2-B50-DR3 (relative risk = 9.4) and A33-DR9 (relative risk = 6.6)\(^14\). It is interesting to note that the A33-B58-DR3 haplotype also shows positive disease-association in Mongoloid racial groups\(^15\). These results support the hypothesis that certain combined haplotypes have subsequently expanded in frequency across populations in different geographical location. The fact that neither A\(^*\)33:03 nor B\(^*\)58:01 appears type 1 diabetes predisposing when seen in Caucasians (e.g., in the T1DGC data) suggests that the effect seen in Asians might not be due to the alleles themselves, but to something else on the haplotype.

The mechanism of the accumulation of specific HLA class I and class II (DR and DQ) alleles causing higher risk in type 1 diabetes remains unclear. The combination of specific HLA-A, -DR and -DQ alleles might have synergic and complementary effects on various steps of the immune response. Furthermore, the odds ratio of A33-DR3 and A33-DR9 was just 5.09 and 13.0, respectively, in new-onset type 1 diabetes, so there remains the possibility that there is synergy between these two haplotypes and non-HLA genes\(^16\) or environmental factors, such as viral infection\(^17\), in promoting insulitis in type 1 diabetes. According to the study by Chang et al.\(^18\), multivariate analysis showed that HLA-A33 was the gene most significantly susceptible to enterovirus 71. There have been several studies to investigate enterovirus’s potential role as an environmental factor in predisposing to type 1 diabetes, though these results remain controversial\(^19-23\).

The significant association of the haplotype A33-DR3 with the abnormality in the ratio of helper to cytotoxic T cells is consistent with our hypothesis. Given that a previous study found that the persistence of a reduced CD4/CD8 lymphocyte ratio (less than 1.5) might reflect the ongoing process leading to β-cell destruction\(^24\), longitudinal observation of β-cell function is necessary to better elucidate the genetic factors that regulate the natural course of β-cell destruction in type 1 diabetes.
Furthermore, as the decreased CD4/CD8 ratio <1 is one of the features leading to the introduction of an immune-risk phenotype (i.e., immunosenesence), the present results suggest that ‘immunosenesence,’ or premature aging of the immune system, could contribute to the development of autoimmune diseases. One surprising finding is that the CD4/CD8 ratio was significantly lower in A33(+) carriers than A33(--), but was not significantly changed between DR3(+) and DR3(--). This point is very interesting compared with the absence of the association of the A33 haplotype alone with type 1 diabetes, and might indicate important roles of HLA class I in immune modulation in the disease.

TCRα/β chains are heterodimeric membrane proteins expressed on the surface of T cells, and they contribute to direct recognition of antigen peptide presented on the major histocompatibility complex in the target cells, so it is reasonable to observe higher TCRα/β T cells in the A33-DR3-positive carriers.

In contrast, none of the serum protein and cells in innate immunity appeared to be associated with the haplotype A33-DR3. According to the study by Diana et al., IgG-producing B cells (CD19+), neutrophils and IFN-γ production in circulation are required for the initiation of the diabetogenic T cell response and type 1 diabetes development. This suggests that repertoire selection in type 1 diabetes could preferentially favor self-reactive T cells, because the HLA-A molecule presents the insulin self-peptide, favoring both a smaller number of surviving T cells and a higher percentage of self-reactivity. In regard to clinical parameters, male sex, a younger age at onset, diabetic ketoacidosis history at onset, a father or sibling with type 1 diabetes and longer duration are independent risk factors for early complete β-cell destruction in type 1 diabetes. However, in the present study, clinical features showed no significant differences between the patients with or without the above-mentioned HLA haplotypes. This might have been due to a narrow range of the age of onset and short duration (less than 6 months) in the present study population as a result of including young patients with new-onset type 1 diabetes, who are mostly in the primary stage of disease.

In a Japanese longitudinal study (median 10 years), patients who possessed HLA-A24, HLA-DQA1*03 and HLA-DR9 together showed complete loss of β-cell function much
earlier than those who did not have this three-allele combination.

Although the present study showed the temporal profile of a disruption of particular immunological cell networks in type 1 diabetes and detected two HLA haplotypes that enhanced the risk of type 1 diabetes, its retrospective design is a limitation with respect to delineating the association between genetic factors and longitudinal changes of residual β-cell function. In addition, as the frequency of A33-DR3 and A33-DR9 was just 19.3% and 6.1%, respectively, in the new-onset type 1 diabetes group, the low frequencies of these haplotypes make it possible that the study was insufficiently powered to find an association with clinical phenotypes. Long-term prospective studies with larger samples on residual β-cell function using the stimulated C-peptide response would be preferable.

This is the first study to report that haplotype A33-DR3 and A33-DR9 were found to predispose patients, and to enhance the risk of type 1 diabetes. Furthermore, A33-DR3 and A33-DR9 were shown to be associated with a reduction in the helper-to-cytotoxic cell ratio (CD4/CD8 ratio) in the initiation of type 1 diabetes. In particular, a preferential increase of TCRα/β cell subpopulations was found in the A33-DR3-positive carriers compared with A33-DR3-negative carriers. In contrast, no relationship was observed between these two haplotypes and the clinical features at onset or the innate immunity. These findings show that typing for HLA class I and its immunogenetic effects are important for more accurate HLA class II haplotype risk prediction and further revelatory etiology research in type 1 diabetic patients.

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DISCLOSURE
The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Sequences of primers for amplifying HLA-A, B, C, DRB1, DQB1 and DQA1.
Table S2 | Sequences of primers for sequencing HLA-A, B, DRB1, DQB1 and DQA1.
Table S3 | Human leukocyte antigen class I allele frequencies in 212 type 1 diabetic patients and 200 people in the control group (positive cases [%]).
Table S4 | Human leukocyte antigen class II allele frequencies in 212 type 1 diabetic patients and 200 people in the control group (positive cases [%]).
Table S5 | Normalized linkage disequilibrium between A*33:03 and DRB1 alleles in type 1 diabetes.
Table S6 | Normalized linkage disequilibrium between B*58:01 and DRB1 alleles in type 1 diabetes.
Table S7 | Normalized linkage disequilibrium between A*33:03 and DQB1 alleles in type 1 diabetes.
Table S8 | Normalized linkage disequilibrium between B*58:01 and DQB1 alleles in type 1 diabetes.
Table S9 | Normalized linkage disequilibrium between A*33:03 and DQA1 alleles in type 1 diabetes.
Table S10 | Normalized linkage disequilibrium between B*58:01 and DQA1 alleles in type 1 diabetes.
Table S11 | Clinical characteristics in patients with high-risk A33-DR3 and A33-DR9 haplotypes.
Figure S1 | Association of A33-DR3 haplotype and T lymphocyte subpopulations (%) in type 1 diabetes patients.
Figure S2 | Association of A33-DR3 haplotype and T lymphocyte subpopulations (×10⁹/L) in type 1 diabetes patients.