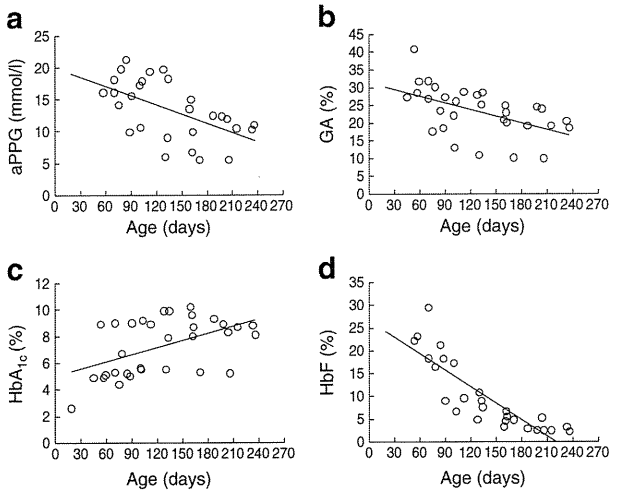

Fig. 2 Time courses of aPPG (**a**), GA (**b**), HbA_{1c} (**c**) and HbF (**d**) for five patients with NDM. (**a**) $r=-0.565$, $p=0.002$; (**b**) $r=-0.552$, $p=0.003$; (**c**) $r=0.449$, $p=0.004$; (**d**) $r=-0.855$, $p<0.0001$

period, the level of HbA_{1c} is low [17] if HbA_{1c} is calculated as a percentage of total haemoglobin, because HbF is the main haemoglobin and HbA accounts for only a small percentage of the total haemoglobin. In addition, most erythrocytes containing HbA in newborns are newly formed after birth and have a relatively short lifespan, which can add to the apparent low value of HbA_{1c}. As a result, HbA_{1c} was measured to be relatively low at the time of NDM diagnosis. However, HbA_{1c} increased despite a decrease in aPPG levels after diabetes treatment. This paradoxical phenomenon may be explained in two ways. First, HbA levels increase as HbF decreases with age. Second, the lifespan of erythrocytes containing HbA increases with age. In patients with NDM, HbA_{1c} levels did not correlate with aPPG, but inversely correlated with HbF. Our findings therefore confirmed that HbA_{1c} is not suitable as a glycaemic control marker for patients with NDM when, as in this study, HbA_{1c} levels are measured by HPLC.

Our study was limited in that we did not compare HbA_{1c} levels obtained from a variety of methods. However, measurements using immunoassay and enzymatic assays tend to yield similar results, because they are both specific assays for HbA_{1c} and do not measure HbF glycosylation products [17]. Although affinity methods can measure total glycated haemoglobins (including both HbA and HbF), glycated haemoglobin levels tend to be measured as low when levels of HbF are high. This is because the glycation rate for HbF, which does not have a glycation site at the N-terminal valine of the gamma chain, may be lower than the glycation rate of HbA [10]. In fact, we found that glycated haemoglobin, measured by an affinity method, did appear low in cord blood [17]. Therefore, these methods should not be used for the assessment of glycation levels in samples from infants. On the other hand, quantification by some HPLC methods (other than those used in this study) showed almost similar HbA_{1c} level in samples with normal to higher than 20% HbF content [10]. These methods were, unfortunately, unavailable to us. There is a possibility that HbA_{1c} may be a useful marker if it is calculated as HbA_{1c}/(total haemoglobin − HbF) in order to eliminate the influence of high HbF levels in neonates. Although we did not examine this issue, the relationship between HbA_{1c}/(total haemoglobin − HbF) and plasma glucose or GA should be examined in future.

Fructosamine and 1,5-anhydroglucitol (1,5-AG), neither of which are affected by haemoglobin metabolism, have also been used as indicators of glycaemic control in adults [21, 22]. However, because 1,5-AG is mainly ingested through food and almost no 1,5-AG is ingested during the neonatal period, serum 1,5-AG levels are undetectable in neonates [23]. Moreover, since fructosamine measures the amount of glycosylated protein, it is influenced by serum protein concentrations [24, 25]. Consequently, low fructosamine levels have been reported during the neonatal period, since serum proteins are low [25]. Moreover, total IgG in neonates, which is derived from maternal IgG, gradually disappears during the first 6–8 months of life. At the same time, the rate of infant IgG synthesis increases [26]. As a result, total IgG usually reaches a low point at 3–4 months of

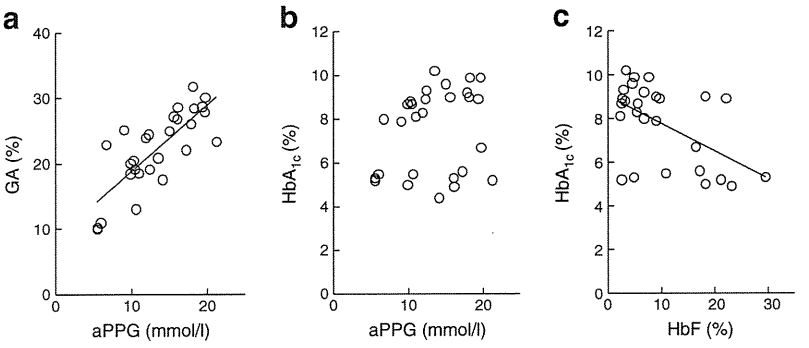

Fig. 3 Correlation of GA (**a**) and HbA_{1c} (**b**) with aPPG and correlation of HbA_{1c} with HbF (**c**) in five patients with NDM. (**a**) $r=0.784$, $p<0.0001$; (**b**) $r=0.221$, $p=0.257$; (**c**) $r=-0.539$, $p=0.004$

age. Because IgG—one of the major components of total protein—is easily glycated [27], the alteration of IgG in infancy may be a large contributor to alterations of fructosamine levels.

GA quantification is not affected by haemoglobin metabolism, nor is it affected by serum albumin concentrations, except in the case of high albumin metabolism, such as that which occurs in nephrotic syndrome [13], since GA is measured against serum albumin as a ratio [13, 16]. In fact, GA is much less affected by serum total protein concentration than is fructosamine [25]. In addition, Shima et al. reported that GA levels are not influenced by the concentration of total serum proteins [28]. In this study, GA was high at the time of NDM diagnosis. With diabetes treatment, GA decreased along with aPPG. Moreover, GA showed a strong positive correlation with aPPG. Based on these results, GA appears to be a very useful marker for both NDM diagnosis and treatment.

GA better reflects short-term changes in plasma glucose than does HbA_{1c} [14, 15]. In patients with fulminant type 1 diabetes, a subtype of type 1 diabetes in which the progression from normoglycaemia to hyperglycaemia accompanied by ketoacidosis is extremely rapid [29], HbA_{1c} levels at onset are normal or only slightly high [30]. On the other hand, GA and GA/HbA_{1c} ratios are already high at disease onset [31]. Therefore, in patients with NDM, in whom hyperglycaemia occurs within a short period from birth to diagnosis, GA is already high at the time of NDM diagnosis—similarly to those individuals with fulminant type 1 diabetes. Moreover, since GA is a shorter-term glycaemic control marker, it may be more useful than a longer-term marker for patients with NDM; it is desirable to monitor glycaemic control changes more frequently in neonates.

We previously reported that the GA values in umbilical cord blood were 9.4±1.1%, which was low compared with the reference values in adults. This may reflect low glucose levels or increased albumin metabolism in the fetus [17]. In this study, GA in infants with NDM was found to be higher than the normal reference values for adults. Abe et al. [25] reported GA reference values for infants, but in that study, GA was measured using HPLC. The use of enzymatic methods, rather than HPLC, is known to provide lower GA values [20], but GA is most commonly measured using an enzymatic method. In the future, GA reference values for infants, measured using enzymatic methods, need to be established.

In conclusion, this study has shown that GA can be a useful indicator of glycaemic control in patients with NDM. These findings will need to be further confirmed in a larger number of patients with NDM.

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S.S. and M.K. researched data, contributed to the discussion, wrote the manuscript, and reviewed/edited the manuscript. S.A., A.N., K.W., K.O., S.H. and A.R.S. researched data and drafted the manuscript. H.T., K.M. and Y.T. contributed to the discussion, and drafted and reviewed/edited the manuscript. K.F. supervised the research. S.S., M.K., S.A., A.N., K.W., K.O., S.H., A.R.S, H.T., K.M. and Y.T. approved the final version.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Original Article

HLA-class II and class I genotypes among Japanese children with Type 1A diabetes and their families

Sugihara S, Ogata T, Kawamura T, Urakami T, Takemoto K, Kikuchi N, Takubo N, Tsubouchi K, Horikawa R, Kobayashi K, Kasahara Y, Kikuchi T, Koike A, Mochizuki T, Minamitani K, Takaya R, Mochizuki H, Nishii A, Yokota I, Kizaki Z, Mori T, Shimura N, Mukai T, Matsuura N, Fujisawa T, Ihara K, Kosaka K, Kizu R, Takahashi T, Matsuo S, Hanaki K, Igarashi Y, Sasaki G, Soneda S, Teno S, Kanzaki S, Saji H, Tokunaga K, Amemiya S and The Japanese Study Group of Insulin Therapy for Childhood and Adolescent Diabetes (JSGIT). HLA-class II and class I genotypes among Japanese children with Type 1A diabetes and their families.
Pediatric Diabetes 2012: 13: 33–44.

Objective: To determine the HLA-DRB1, DQB1, DPB1, A, C, and B genotypes among Japanese children with autoimmune type 1 diabetes.
Methods: Four hundred and thirty patients who were GADAb and/or IA-2Ab-positive (Type 1A) were recruited from 37 medical centers as part of a nationwide multicenter collaborative study. DNA samples from 83 siblings of the children with Type 1A diabetes and 149 parent–child trios were also analyzed. A case-control study and a transmission disequilibrium test (TDT) were then performed.
Results: The susceptible and protective DRB1 and DQB1 alleles and haplotypes were confirmed. DPB1 alleles unique to the Japanese population and those common to multiple ethnic groups were also present. A linkage disequilibrium (LD) analysis showed both susceptible and protective haplotypes. The TDT did not reveal any alleles that were transmitted preferentially from the mother or father to children with Type 1A. Homozygosity for DRB1*09:01-DQB1*03:03 and heterozygosity for DRB1*04:05-DQB1*04:01 and DRB1*08:02-DQB1*03:02 were associated with an extremely high risk of Type 1A. A comparison of children with Type 1A and their parents and siblings suggested a dose effect of susceptible DRB1-DQB1 haplotypes and an effect of protective alleles on immunological pathogenesis. DRB1*09:01 appeared to be strongly associated with an early onset in preschool children with Type 1A diabetes.
Conclusions: This study demonstrated the characteristic association of HLA-class II and class I genes with Type 1A diabetes among Japanese children. A TDT did not reveal the genomic imprinting of HLA-class II and class I genes in Type 1A diabetes.

Genetic and environmental factors are thought to be responsible for differences in the incidence of type 1 diabetes among different ethnic groups. The contribution of the HLA-DRB1, DQA1, and DQB1 genes to susceptibility to autoimmune type 1 diabetes (Type 1A) has been well described (1, 2). Several genome scans for linkage to type 1 diabetes have been performed, and these studies have indicated that a gene or genes in the HLA region (insulin-dependent diabetes mellitus 1) at 6p21 has or have

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the strongest impact on disease risk (2, 3). In addition, the independent effects of HLA-DPB1, A, and B have also been demonstrated (4, 5).

The incidence of childhood-onset type 1 diabetes mellitus in Japan is very low (1.4–2.2/100 000 individuals per year) compared with Caucasian populations, especially in Europe (Sardinia, Finland, Sweden, and the UK) and Canada (20/100 000 per year) (6). The risk for siblings of individuals with type 1 diabetes is similar between Caucasians (about 6%) and Japanese (3.8%) (7, 8). These results suggest the existence of both a different set of immunogenetic mechanisms in Japanese patients with type 1 diabetes and a common pathogenesis with Caucasian patients.

The genetic effects of HLA-DRB1 and DQB1 in Japanese patients with type 1 diabetes reportedly differ from those in Caucasian patients (9–15). In Caucasian populations, a predisposition to type 1 diabetes is mostly associated with the DRB1*03:01-DQA1*05:01-DQB1*02:01 and/or DRB1*04:01-DQA1*03:01-DQB1*03:02 haplotypes, whereas the DRB1*15:01-DQB1*06:02 haplotype confers strong protection against the disease. In the Japanese population, three characteristic haplotypes confer susceptibility to type 1 diabetes: DRB1*04:05-DQB1*04:01, DRB1*08:02-DQB1*03:02, and DRB1*09:01-DQB1*03:03. Furthermore, two haplotypes confer protection: DRB1*15:01-DQB1*06:02 (which is common among Caucasians), and DRB1*15:02-DQB1*06:01 (which is characteristic of the Japanese population) (11–15).

HLA-DPB1 alleles are not generally recognized as major contributors to type 1 diabetes. However, an increased risk associated with allele DPB1*02:02 and *03:01 and a decreased risk associated with allele *04:02 have been reported in a number of ethnic groups (4, 5, 16–19). The association of DPB1*02:01 with Japanese childhood-onset type 1 diabetes has been reported by Nishimaki et al. (20), but the number of subjects in this study was relatively small.

This study is the first nationwide multicenter collaborative study for genetic factors in Japanese children with type 1 diabetes and their families. The objective of this study was to determine the genetic characteristics of both HLA-class II (DRB1, DQB1, and DPB1), and class I (A, C, and B) genotypes among Japanese children with Type 1A diabetes and to compare these characteristics with both control data and data obtained from the parents and siblings of the children with Type 1A diabetes. We also studied the diabetes-associated allelic transmission rates from mothers and fathers to children with Type 1A diabetes in the Japanese population.

Methods

Subjects

We recruited 497 Japanese children with type 1 diabetes from 37 medical centers throughout Japan between February 2008 and February 2009. The patients were divided into two groups: Type 1A (GADAb and/or IA-2Ab-positive at diagnosis and/or at registration in this study) and Type 1B (GADAb and IA-2Ab-negative). Type 1A accounted for 430 patients (158 boys and 272 girls) who were 0.8–16.4 years old (mean ± SD, 7.6 ± 3.7 years) at the time of diagnosis. Type 1B accounted for 67 patients (28 boys and 39 girls) who were 0.1–15.1 years old (6.2 ± 4.4 years) at the time of diagnosis. In this study, we focused on children with Type 1A diabetes. Type 1B diabetes may have heterogeneous pathogenetic mechanisms, and some cases of Type 1B have been shown to have a particular monogenic cause, such as mutations in the insulin gene (*INS*), *KCNJ11*, or *ABCC8*. Furthermore, the number of subjects with Type 1B diabetes was too small to obtain a sufficient power in the case-control study.

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Clinical data for all the type 1 diabetes children were obtained. The diagnosis of type 1 diabetes was based on both clinical features and laboratory data. All the patients with Type 1A diabetes were ketosis-prone, lacked endogenous insulin secretion, and required insulin injections at the time of diagnosis based on the 1999 Japan Diabetes Society criteria. The HbA1c levels at the time of diagnosis were 11.9 ± 2.6% among the patients with Type 1A diabetes. The insulin dose at the time of study registration was 1.1 ± 0.3 units/kg/day among the patients with Type 1A diabetes. Eighty-three siblings of 66 children with Type 1A diabetes and 148 father and mother pairs of 149 children with Type 1A diabetes (149 parent–child trios) were recruited. The control data for the HLA allele and haplotype frequencies were based on previously reported data for 1216 subjects in a general Japanese population (21) and a study of 159 families with 561 subjects (22).

This study was approved by the institutional ethics review board of the Tokyo Women's Medical University, the National Research Institute for Child Health and Development, and each of the clinics or hospitals affiliated with a study collaborator. Written informed consent was obtained from the parents or guardians and/or the participants.

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Key words: genetic factors – HLA – Japanese children – type 1 diabetes

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HLA typing

Genomic DNA was extracted from whole blood samples. HLA typing was performed using a Luminex Multi-Analyte Profiling system with a WAKFlow HLA typing Kit (Wakunaga, Hiroshima, Japan), as described elsewhere (23). Briefly, highly polymorphic exons 2 and 3 of the HLA-A, -B, and -C genes and exon 2 of the HLA-DRB1, -DQB1, and -DPB1 genes were amplified using the primer pairs included with the kit. Each polymerase chain reaction product was hybridized using sequence-specific oligonucleotide probes that were complementary to the allele-specific sequences.

Statistical analysis

All the statistical analyses were performed using the R statistical environment, version 2.9.1 (http://www.r-project.org/). The Fisher exact test was applied to a two-by-two contingency table, and the corrected p values (Pc), equivalent to the p values multiplied by the number of comparisons for each locus or haplotype, were determined. A Pc value <0.05 was considered statistically significant.

The study had a sufficient power (more than 0.98) to detect an odds ratio (OR) = 2.0 for an allele frequency of 0.1 in the case-control study comparing DRB1, DQB1, DPB1, A, C, and B between the children with Type 1A diabetes ($n = 430$) and the control data.

The frequency of HLA haplotypes was estimated using the maximum likelihood method (24) or the PHASE program (25). Relative linkage disequilibrium (RD) was calculated as the linkage disequilibrium (LD)/|Dmax| for the relative assessment of LD (22). |Dmax| was the absolute value of the maximum LD for the haplotype.

Results

Association of HLA-DRB1, DQB1, and DPB1 with Type 1A diabetes

In the case-control study, the susceptible alleles associated with Type 1A diabetes in Japanese children were DRBI*09:01 ($Pc < 10^{-29}$; OR, 3.00), DRBI*04:05 ($Pc < 10^{-20}$; OR, 2.60), DRBI*08:02 ($Pc < 10^{-12}$; OR, 3.11), DQBI*03:03 ($Pc < 10^{-26}$; OR, 2.80), DQBI*04:01 ($Pc < 10^{-16}$; OR, 2.32), DQBI*03:02 ($Pc < 10^{-12}$; OR, 2.34), DPBI*02:01 ($Pc < 10^{-2}$; OR, 1.49), and DPBI*03:01 ($Pc < 0.05$; OR, 1.92). The protective alleles were DRBI*15:02 ($Pc < 10^{-21}$; OR, 0.09), DRBI*15:01 ($Pc < 10^{-16}$; OR, 0.06), DRBI*08:03 ($Pc < 10^{-14}$; OR, 0.14), DRBI*04:06 ($Pc < 10^{-3}$; OR, 0.23), DQBI*06:01 ($Pc < 10^{-36}$; OR, 0.11), DQBI*06:02 ($Pc < 10^{-19}$; OR, 0.00), DQBI*03:01 ($Pc < 10^{-11}$; OR, 0.29), DPBI*09:01 ($Pc < 10^{-8}$; OR, 0.25), and DPBI*04:02 ($Pc < 10^{-2}$; OR, 0.57) (Table 1).

The susceptible HLA-DRB1-DQB1 haplotypes associated with Type 1A diabetes in Japanese children were DRB1*09:01-DQBI*03:03 ($Pc < 10^{-20}$; OR, 3.05), DRB1*04:05-DQBI*04:01 ($Pc < 10^{-10}$; OR, 2.33), DRB1*08:02-DQBI*03:02 ($Pc < 10^{-11}$; OR, 5.41), and DRB1*04:05-DQBI*03:02 ($Pc < 10^{-11}$). The protective HLA-DRB1-DQB1 haplotypes were

Table 1. HLA-DRB1, DQB1, and DPB1 allele frequencies among Japanese children with Type 1A diabetes

		Type 1A		Control		Type 1A vs. Control		
HLA	Allele	n = 860	%	n	%	Pc	OR	(95% CI)
DRB1	*04:05	244	28.37	322	13.26	$<10^{-20}$	2.60	(2.15–3.14)
	*08:02	103	11.98	102	4.18	$<10^{-12}$	3.11	(2.34–4.14)
	*09:01	283	32.91	342	14.08	$<10^{-29}$	3.00	(2.50–3.60)
	*04:06	6	0.70	73	3.00	$<10^{-3}$	0.23	(0.10–0.52)
	*08:03	11	1.28	202	8.29	$<10^{-14}$	0.14	(0.08–0.26)
	*15:01	4	0.47	173	7.11	$<10^{-16}$	0.06	(0.02–0.16)
	*15:02	9	1.05	246	10.13	$<10^{-21}$	0.09	(0.05–0.18)
	*13:02	39	4.53	166	6.83	NS		
	Others	161	18.72	806	33.14			
DQB1	*03:02	167	19.42	227	9.32	$<10^{-12}$	2.34	(1.88–2.91)
	*03:03	282	32.79	361	14.86	$<10^{-26}$	2.80	(2.34–3.35)
	*04:01	222	25.81	317	13.03	$<10^{-15}$	2.32	(1.91–2.82)
	*03:01	31	3.60	282	11.61	$<10^{-11}$	0.29	(0.20–0.42)
	*06:01	21	2.44	440	18.11	$<10^{-36}$	0.11	(0.07–0.18)
	*06:02	0	0.00	151	6.22	$<10^{-19}$	0.00	
	*06:04	37	4.30	167	6.88	NS		
	Others	100	11.63	486	19.98			
DPB1	*02:01	244	28.37	273	21.02	$<10^{-2}$	1.49	(1.22–1.82)
	*03:01	59	6.86	48	3.68	<0.05	1.92	(1.30–2.84)
	*04:02	53	6.16	135	10.40	$<10^{-2}$	0.57	(0.41–0.79)
	*09:01	21	2.44	118	9.12	$<10^{-8}$	0.25	(0.16–0.40)
	*04:01	42	4.88	49	3.80	NS		
	Others	433	50.35	647	26.60			

CI, confidence interval; n, total number of alleles; Pc, corrected p values; OR, odds ratio; NS, not significant.
The total number of alleles in the control data for DRB1 and DQB1 was 2432, while the total number of alleles in the control data for DPB1 was 1298 (21).
Others for DRB1: *01:01, *03:01, *04:01, *04:03, *04:04, *04:07, *04:10, *07:01, *10:01, *11:01, *11:05, *11:06, *12:01, *12:02, *14:01, *14:03, *14:06, *16:02.
Others for DQB1: *02:01, *04:02, *05:01, *05:02, *06:09.
Others for DPB1: *01:01, *02:02, *05:01, *06:01, *13:01, *14:01, *17:01, *19:01, *25:01, *26:01, *29:01, *38:01, *41:01, *48:01.
Corrected p values (Pc), or the p values multiplied by the number of comparisons at each locus, are shown. A Pc value < 0.05 was considered significant.

DRB1*15:01-DQBI*06:02 ($Pc < 10^{-31}$; OR, 0.0), DRB1*15:02-DQBI*06:01 ($Pc < 10^{-14}$; OR, 0.11), and DRB1*08:03-DQBI*06:01 ($Pc < 10^{-6}$; OR, 0.18) (Table 2).

In the transmission disequilibrium test (TDT), the susceptible alleles associated with Type 1A diabetes in Japanese children were DRBI*04:05 ($Pc < 10^{-5}$; OR, 2.83), DRBI*09:01 ($Pc < 10^{-5}$; OR, 2.58), DRBI*08:02 ($Pc < 10^{-3}$; OR, 5.33), DQBI*04:01 ($Pc < 10^{-5}$; OR, 2.76), DQBI*03:03 ($Pc < 10^{-5}$; OR, 2.69), and DQBI*03:02 ($Pc < 10^{-3}$; OR, 2.88) (Table 3). DPB1*02:01 and DPB1*03:01 were not significant when examined using the TDT. The protective alleles were DRBI*15:02 ($Pc < 10^{-6}$; OR, 0.08), DRBI*15:01 ($Pc < 10^{-5}$; OR, 0.00), DRBI*08:03 ($Pc < 0.05$; OR, 0.26), DQBI*06:01 ($Pc < 10^{-9}$; OR, 0.13), DQBI*06:02 ($Pc < 10^{-5}$; OR, 0.00), DQBI*03:01 ($Pc < 10^{-4}$; OR, 0.18), and DPBI*09:01 ($Pc < 10^{-4}$; OR, 0.20); DRB1*04:06 and DPB1*04:02 were not significant when examined using the TDT (Table 3).

Association of HLA-A, C, and B with Type 1A diabetes

In the case-control study, the susceptible alleles associated with Type 1A diabetes in Japanese children were A*24:02 ($Pc < 10^{-2}$; OR, 1.44), C*01:02 ($Pc < 10^{-2}$; OR, 1.56), C*08:01 ($Pc < 0.05$; OR, 1.60), B*07:02 ($Pc < 10^{-3}$; OR, 2.39), B*40:06 ($Pc < 10^{-3}$; OR, 2.21), and B*54:01 ($Pc < 10^{-10}$; OR, 2.82). The protective alleles were A*26:01 ($Pc < 10^{-4}$; OR, 0.43), A*33:03 ($Pc < 10^{-2}$; OR, 0.47), A*11:01 ($Pc < 0.05$; OR, 0.60), C*12:02 ($Pc < 10^{-8}$; OR, 0.28), C*14:03 ($Pc < 10^{-3}$; OR, 0.41), C*15:02 ($Pc < 10^{-3}$; OR, 0.28), B*15:01 ($Pc < 10^{-6}$; OR, 0.30), B*52:01 ($Pc < 10^{-9}$; OR, 0.26), and B*44:03 ($Pc < 0.05$; OR, 0.47) (Table 4).

In the TDT, the susceptible alleles associated with Type 1A diabetes in Japanese children were C*01:02 ($Pc < 10^{-2}$; OR, 1.92), C*08:01 ($Pc < 0.05$; OR, 2.15), and B*54:01 ($Pc < 10^{-5}$; OR, 4.13) (Table 3). The protective alleles were A*33:03 ($Pc < 10^{-2}$; OR, 0.32),

Table 2. Haplotype frequencies of HLA-DRB1-DQB1 among Japanese children with Type 1A diabetes

HLA haplotype	Type 1A		Control			Type 1A vs. Control		
DRB1-DQB1	n = 860	%	n = 1032	%	p	Pc	OR	(95% CI)
*09:01-*03:03	275	31.98	138	13.37	2.19E-22	$<10^{-20}$	3.05	(2.42–3.83)
*04:05-*04:01	222	25.81	134	12.98	1.43E-12	$<10^{-10}$	2.33	(1.84–2.96)
*08:02-*03:02	83	9.65	20	1.94	1.01E-13	$<10^{-11}$	5.41	(3.29–8.89)
*04:05-*03:02	35	4.07	0	0.00	7.05E-13	$<10^{-11}$		
*08:03-*06:01	10	1.16	62	6.01	9.35E-09	$<10^{-6}$	0.18	(0.09–0.36)
*15:02-*06:01	9	1.05	92	8.91	4.24E-16	$<10^{-14}$	0.11	(0.05–0.22)
*15:01-*06:02	0	0.00	118	11.43	3.75E-33	$<10^{-31}$	0.00	
*04:07-*03:02	14	1.63	4	0.39	7.45E-03	NS		
*01:01-*05:01	25	2.91	40	3.88	NS			
*13:02-*06:04	37	4.30	56	5.43	NS			
*15:01-*03:01	4	0.47	2	0.19	NS			
Others	146	16.98	366	35.47				

CI, confidence interval; n, total number of alleles; OR, odds ratio.
The control data were obtained from Ref. (22).

C*12:02 ($Pc < 10^{-5}$; OR, 0.18), C*14:03 ($Pc < 0.05$; OR, 0.33), B*15:01 ($Pc < 0.05$; OR, 0.34), and B*52:01 ($Pc < 10^{-5}$; OR, 0.17) (Table 3).

Linkage disequilibrium (LD) between DRB1-DQB1 haplotypes and DPB1, A, C, or B alleles

DPB1*02:01 and *03:01 were assessed as susceptible alleles in the case-control study but were not specifically associated with any susceptible DRB1-DQB1 haplotype. The RD values for DPB1*02:01 to DRB1*04:05-DQB1*04:01, DRB1*09:01-DQB1*03:03, and DRB1*08:02-DQB1*03:02 were 0.246, 0.312, and 0.112, respectively. The RD values for DPB1*03:01 to DRB*04:05-DQB1*04:01, DRB1*09:01-DQB1*03:03, and DRB1*08:02-DQB1*03:02 were 0.175, 0.081, and 0.148, respectively. A*24:02 was assessed as a susceptible allele in the case-control study but was not specifically associated with any susceptible DRB1-DQB1 haplotype. The RD values for A*24:02 to DRB1*04:05-DQB1*04:01, DRB1*09:01-DQB1*03:03, and DRB1*08:02-DQB1*03:02 were 0.405, 0.310, and 0.286, respectively. However, the susceptible alleles C*01:02 and B*54:01 appeared to be associated with the DRB1*04:05-DQB1*04:01 haplotype (RD, 0.697). Meanwhile, the susceptible C*08:01 and B*40:06 alleles appeared to be associated with the DRB1*09:01-DQB1*03:03 haplotype (RD, 0.597).

DPB1*09:01, C*12:02, and B*52:01 were assessed as protective alleles in both the case-control study and the TDT and appeared to be associated with the protective DRB1*15:02-DQB1*06:01 haplotype. The RD for the C*12:02-B*52:01-DRB1*15:02-DQB1*06:01-DPB1*09:01haplotype was 0.861 among the Japanese children with Type 1A diabetes in this study. The protective alleles A*33:03, C*14:03, and B*44:03 were associated with a high LD (RD, 0.842).

Transmission of susceptible and protective alleles from maternal and paternal parents

In the TDT, the transmission of DRB1*08:02 from the father occurred more frequently than from the mother, but the difference was not significant. The transmission of DRB1*09:01 from the mother occurred more frequently than from the father, but again the difference was not significant. The DRB1, DQB1, and DPB1 alleles were not transmitted preferentially from the mother or father to the children with Type 1A diabetes (Table 3), and the same was true for the A, C, and B alleles (Table 3).

Comparison of combinations of susceptible haplotypes and protective alleles between children with Type 1A diabetes and their parents

When genetic combinations of HLA-DRB1-DQB1 haplotypes were compared between children with Type 1A diabetes and their parents (149 parent–child trios), 54.4% of the children with Type 1A diabetes and 21.3% of their parents had two susceptible haplotypes. The frequencies of DR9/9 (homozygotes for DRB1*09:01-DQB1*03:03) ($Pc < 10^{-2}$; OR, 3.77) in group I (homozygotes for two susceptible haplotypes) and DR4/8 (heterozygotes for DRB1*04:05-DQB1*04:01 and DRB1*08:02-DQB1*03:02) ($Pc < 10^{-2}$; OR, 4.38) in group II (heterozygotes for two susceptible haplotypes) were significantly higher among the children with Type IA diabetes. The frequencies of group IV (one susceptible haplotype and a protective allele) ($Pc < 10^{-10}$; OR, 0.16) and group VI (no susceptible haplotypes and a

Table 3. Transmission disequilibrium test (TDT) for HLA-DRB1, DQB, DPB1, A, C, and B alleles in 149 parent–child trios

		Transmitted			Non-transmitted			TDT				Transmission from Maternal vs. Paternal
			Parent of origin			Parent of origin						
HLA		Combined	Maternal	Paternal	Combined	Maternal	Paternal	P	Pc	OR	(95% CI)	p
DRB1	*04:05	85	41	44	30	12	18	2.92E-07	$<10^{-5}$	2.83	(1.87–4.30)	NS
	*08:02	32	13	19	6	5	1	2.47E-05	$<10^{-3}$	5.33	(2.23–12.76)	NS
	*09:01	85	47	38	33	13	20	1.69E-06	$<10^{-5}$	2.58	(1.72–3.85)	NS
	*08:03	6	1	5	23	13	10	1.59E-03	<0.05	0.26	(0.11–0.64)	NS
	*15:01	0	0	0	26	15	11	3.41E-07	$<10^{-5}$	0.00		NS
	*15:02	3	1	2	39	21	18	2.78E-08	$<10^{-6}$	0.08	(0.02–0.25)	NS
	*04:06	2	2	0	10	4	6	2.09E-02	NS			NS
DQB1	*04:01	80	38	42	29	12	17	1.03E-06	$<10^{-5}$	2.76	(1.80–4.22)	NS
	*03:02	49	28	21	17	9	8	8.18E-05	$<10^{-3}$	2.88	(1.66–5.00)	NS
	*03:03	86	46	40	32	15	17	6.66E-07	$<10^{-5}$	2.69	(1.79–4.03)	NS
	*03:01	7	4	3	40	17	23	1.48E-06	$<10^{-4}$	0.18	(0.08–0.39)	NS
	*06:01	8	2	6	62	34	28	1.09E-10	$<10^{-9}$	0.13	(0.06–0.27)	NS
	*06:02	0	0	0	25	15	10	5.73E-07	$<10^{-5}$	0.00		NS
DPB1	*02:01	63	30	33	43	17	26	NS				NS
	*03:01	23	12	11	14	8	6	NS				NS
	*09:01	7	3	4	35	18	17	1.56E-05	$<10^{-4}$	0.20	(0.09–0.45)	NS
	*04:02	21	10	11	24	14	10	NS				NS
A	*24:02	100	48	52	70	36	34	2.14E-02	NS			NS
	*33:03	9	4	5	28	17	11	1.79E-03	$<10^{-2}$	0.32	(0.15–0.68)	NS
	*11:01	19	11	8	26	15	11	NS				NS
	*26:01	14	9	5	20	10	10	NS				NS
C	*01:02	69	30	39	36	18	18	1.28E-03	$<10^{-2}$	1.92	(1.28–2.87)	NS
	*08:01	43	22	21	20	11	9	3.76E-03	<0.05	2.15	(1.26–3.65)	NS
	*12:02	7	4	3	40	21	19	1.48E-06	$<10^{-5}$	0.18	(0.08–0.39)	NS
	*14:03	8	4	4	24	14	10	4.68E-03	<0.05	0.33	(0.15–0.74)	NS
	*15:02	7	4	3	13	8	5	NS				NS
B	*54:01	62	27	35	15	7	8	8.50E-08	$<10^{-5}$	4.13	(2.35–7.26)	NS
	*40:06	28	14	14	12	3	9	1.14E-02	NS			NS
	*07:02	17	7	10	12	5	7	NS				NS
	*15:01	10	6	4	29	14	15	2.35E-03	<0.05	0.34	(0.17–0.71)	NS
	*52:01	7	4	3	42	23	19	5.73E-07	$<10^{-5}$	0.17	(0.07–0.37)	NS
	*44:03	9	5	4	24	14	10	9.02E-03	NS			NS

CI, confidence interval; OR, odds ratio; TDT, transmission disequilibrium test.

Table 4. HLA-A, C, and B allele frequencies among Japanese children with Type 1A diabetes

HLA		Type 1A		Control		Type 1A vs. Control		
		n = 860	%	n = 1046	%	Pc	OR	(95% CI)
A	*24:02	390	45.35	382	36.52	$<10^{-2}$	1.44	(1.20–1.73)
	*26:01	45	5.23	118	11.28	$<10^{-4}$	0.43	(0.30–0.62)
	*33:03	33	3.84	82	7.84	$<10^{-2}$	0.47	(0.31–0.71)
	*11:01	58	6.74	112	10.71	<0.05	0.60	(0.43–0.84)
	Others	334	38.84	352	33.65			
C	*01:02	204	23.72	174	16.63	$<10^{-2}$	1.56	(1.24–1.95)
	*08:01	127	14.77	102	9.75	<0.05	1.60	(1.21–2.12)
	*12:02	29	3.37	116	11.09	$<10^{-8}$	0.28	(0.18–0.42)
	*14:03	26	3.02	74	7.07	$<10^{-3}$	0.41	(0.26–0.65)
	*15:02	11	1.28	46	4.40	$<10^{-3}$	0.28	(0.14–0.55)
	Others	450	52.33	534	51.05			
B	*07:02	71	8.26	38	3.63	$<10^{-3}$	2.39	(1.59–3.58)
	*40:06	86	10.00	50	4.78	$<10^{-3}$	2.21	(1.54–3.18)
	*54:01	152	17.67	74	7.07	$<10^{-10}$	2.82	(2.10–3.78)
	*15:01	26	3.02	98	9.37	$<10^{-6}$	0.30	(0.19–0.47)
	*52:01	27	3.14	114	10.9	$<10^{-9}$	0.26	(0.17–0.41)
	*44:03	28	3.26	70	6.69	<0.05	0.47	(0.30–0.73)
	Others	470	54.65	602	57.55			

CI, confidence interval; n, total number of alleles; OR, odds ratio.
The control data was obtained from Ref. (22).
Others for A: *01:01, *02:01. *02:06, *02:07, *02:10, *11:02, *24:02, *24:08, *26:02, *26:03, *26:05, *31:01, *32:01.
Others for C: *03:02, *03:03, *03:04, *04:01, *05:01, *06:02, *07:02, *07:04, *08:03, *14:02.
Others for B: *08:01, *13:01, *13:02, *15:02, *15:07, *15:11, *15:18, *27:04, *35:01, *37:01, *38:01, *39:01, *39:02, *39:04, *40:01, *40:02, *40:03, *46:01, *48:01, *51:01, *54:12, *55:02, *55:04, *56:01, *58:01, *59:01, *67:01.

protective allele) (Pc < 0.05; OR, 0.20) were significantly lower among the children with Type 1A diabetes than among their parents (Table 5). Of note, the frequency of group III (one susceptible haplotype and no protective allele) was similar between the children with Type 1A diabetes and their parents (Table 5).

GADAb and/or IA-2Ab were positive in 21 (7.1%) of the 296 parents: one in group I, five in group II, six in group III, four in group IV, three in group V, and two in group VI. Three parents (1.0%) had type 1 diabetes mellitus: two in group II and one in group III.

Comparison between children with Type 1A diabetes and their siblings

When the frequencies of the HLA-DRB1, DQB1, and DPB1 alleles were compared between 66 children with Type 1A diabetes and their 83 healthy siblings, the prevalences of all the alleles except for DQB1*06:01 were not significantly different. The frequency of the DQB1*06:01 protective allele was lower (Pc < 10^{-2}; OR, 0.13) among the patients than among their siblings.

When genetic combinations of HLA-DRB1-DQB1 haplotypes were compared between children with Type 1A diabetes and their siblings, the frequency of group VI (no susceptible haplotypes and a protective allele) was lower (Pc < 10^{-2}; OR, 0.09) among the children with Type 1A diabetes (3.03%) than among the siblings (25.3%) (Table 6). Of note, 44.6% of the siblings had protective alleles (groups IV + VI), compared with 10.6% of the children with Type 1A diabetes.

GADAb and/or IA-2Ab were positive in 7 (8.4%) of the 83 siblings: three in group II, three in group III, and one in group V. Groups II, III, and V can be characterized as having no protective alleles.

Onset age and HLA genotype

The DRB1 allele frequencies in four age groups, determined according to the patient's age at the time of Type 1A diabetes onset (0–1, 2–5, 6–9, and 10–16 years), are shown in Fig. 1. The frequency of DRB1*09:01 was higher (Pc < 0.01) in the 2–5-year onset group than in the other age groups, while the frequency of DRB1*08:02 tended to be higher in the 6–16-year onset group, although the difference was not significant (Fig. 1). The distribution of the DRB1*04:05 allele was not different among the four age groups. The distributions of other alleles, including DPB1*02:01, DPB1*03:01, A*24:02, C*01:02, C*08:01, and B*54:01, were not different among the four age groups (data not shown).

Discussion

This study is the first nationwide multicenter collaborative study examining genetic factors associated with

Table 5. Genetic combinations of HLA-DRB1-DQB1 haplotypes in Japanese children with Type 1A diabetes and their parents

Genetic combination of	Type 1A all		Type 1A in trio		Parents in trio		Type 1A in trio vs. Parents			
HLA-DRB1-DQB1 haplotype	n = 430	%	n = 149	%	n = 296	%	p	Pc	OR	(95% CI)
I. Two susceptible haplotypes in homozygote	82	19.07	37	24.83	21	7.09	4.33E-07	$<10^{-5}$	4.33	(2.43–7.72)
DR4/4 (*04:05-*04:01)	8	1.86	7	4.70	5	1.69	NS			
DR4/4 (*04:05-*03:02)	10	2.33	6	4.03	2	0.68	1.92E-02	NS		
DR9/9 (*09:01-*03:03)	58	13.49	22	14.77	13	4.39	2.68E-04	$<10^{-2}$	3.77	(1.84–7.72)
DR8/8 (*08:02-*03:02)	6	1.40	2	1.34	1	0.34	NS			
II. Two susceptible haplotypes in heterozygote	143	33.26	44	29.53	42	14.19	1.96E-04	$<10^{-2}$	2.53	(1.57–4.10)
DR4/9	65	15.12	19	12.75	26	8.78	NS			
DR4/8	61	14.19	18	12.08	9	3.04	4.59E-04	$<10^{-2}$	4.38	(1.92–10.01)
DR9/8	17	3.95	7	4.70	7	2.36	NS			
III. One susceptible haplotype and no protective allele	135	31.40	44	29.53	66	22.30	NS			
DR4/X	62	14.42	23	15.44	23	7.77	1.99E-02	NS		
DR9/X	64	14.88	18	12.08	32	10.81	NS			
DR8/X	9	2.09	3	2.01	10	3.38	NS			
IV. One susceptible haplotype and a protective allele	43	10.00	15	10.07	121	40.88	2.62E-12	$<10^{-10}$	0.16	(0.09–0.29)
V. No susceptible haplotype and no protective allele	13	3.02	6	4.03	19	6.42	NS			
VI. No susceptible haplotype and a protective allele	14	3.26	3	2.01	28	9.46	2.66E-03	<0.05	0.20	(0.06–0.66)

CI, confidence interval; OR, odds ratio.
Susceptible haplotype: *04:05-*04:01, *09:01-*03:03, *08:02-*03:02, *04:05-*03:02.
Protective allele in DRB1: *08:03, *15:01, *15:02, *04:06.
Protective allele in DQB1: *06:01, *06:02, *03:01.
X in DRB1: *01:01, *03:01, *04:01, *04:03,*04:04,*04:07, *04:10, *07:01,*10:01, *11:01, *11:06, *12:01, *12:02, *13:02, *16:02.
X in DQB1: *02:01, *04:02, *05:01, *05:02, *06:04, *06:09.

Table 6. Genetic combinations of HLA-DRB1-DQB1 haplotypes in Japanese children with Type 1A diabetes and their siblings

Genetic combination of HLA-DRB1-DQB1 haplotype	Type 1A n = 66	%	Siblings n = 83	%	Type 1A vs. Siblings p	Pc	OR	(95% CI)
I. Two susceptible haplotypes in homozygote	16	24.24	11	13.25	NS			
DR4/4 (*04:05-*04:01)	4	6.06	3	3.61	NS			
DR4/4 (*04:05-*03:02)	3	4.55	2	2.41	NS			
DR9/9 (*09:01-*03:03)	9	13.64	6	7.23	NS			
DR8/8 (*08:02-*03:02)	0	0.00	0	0.00	NS			
II. Two susceptible haplotypes in heterozygote	19	28.79	17	20.48	NS			
DR4/9	9	13.64	13	15.66	NS			
DR4/8	8	12.12	2	2.41	2.31E-02	NS		
DR9/8	2	3.03	2	2.41	NS			
III. One susceptible haplotype and no protective allele	20	30.30	15	18.07	NS			
DR4/X	9	13.64	5	6.02	NS			
DR9/X	10	15.15	7	8.43	NS			
DR8/X	1	1.52	3	3.61	NS			
IV. One susceptible haplotype and a protective allele	5	7.58	16	19.28	NS			
V. No susceptible haplotype and no protective allele	4	6.06	3	3.61	NS			
VI. No susceptible haplotype and a protective allele	2	3.03	21	25.30	1.50E-04	$<10^{-2}$	0.09	(0.02–0.41)

CI, confidence interval; OR, odds ratio.
Susceptible haplotype: *04:05-*04:01, *09:01-*03:03, *08:02-*03:02, *04:05-*03:02.
Protective allele in DRB1: *08:03, *15:01, *15:02, *04:06.
Protective allele in DQB1: *06:01, *06:02, *03:01.
X in DRB1: *01:01, *03:01, *04:01, *04:03,*04:04,*04:07, *04:10, *07:01,*10:01, *11:01, *11:06, *12:01, *12:02, *13:02, *16:02.
X in DQB1: *02:01, *04:02, *05:01, *05:02, *06:04, *06:09.

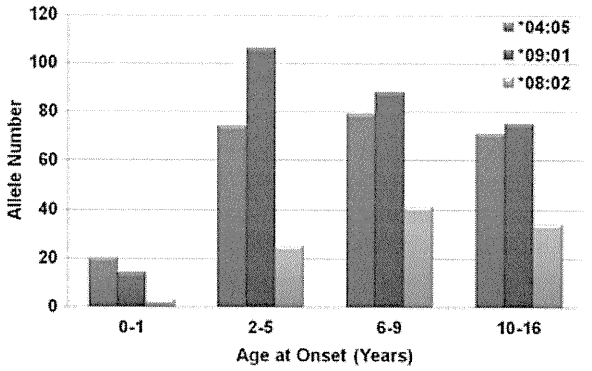

Fig. 1. DRB1 allele frequencies in four age groups of Japanese children with Type 1A diabetes according to the age at onset. The frequency of DRB1*09:01 was higher (Pc < 0.01) in the 2–5-year onset group, while the frequency of DRB1*08:02 tended to be higher in the 6–16-year onset group, although the difference was not significant. The distribution of the DRB1*04:05 allele frequency was not different among the four age groups.

childhood-onset type 1 diabetes mellitus in Japan. In the study, a TDT was performed for the first time in a population of Japanese children with Type 1A diabetes; the results confirmed the identities of susceptible and protective DRB1, DQB1, DPB1, A, C, and B, alleles.

We confirmed previously reported HLA-DRB1-DQB1 susceptible and protective haplotypes and obtained new findings regarding the DPB1 allele. DPB1*02:01 and DPB1*03:01 were identified as susceptible alleles among Japanese children with Type 1A diabetes (Table 1). DPB1*02:01 is unique to the Japanese population, while DPB1*03:01 is observed in multiple ethnic groups (4, 5, 16–19). This finding is noteworthy because neither the susceptible DRB1 allele nor the DQB1 allele is common to both Japanese and Caucasian populations. Moreover, the DPB1*02:01 and DPB1*03:01 alleles were not specifically associated with any susceptible DRB1-DQB1 haplotype. DPB1*04:02 was identified as a protective allele among Japanese children with Type 1A diabetes, similar to cases in multiple ethnic groups (4, 5, 16–19). Moreover, the DPB1*04:02 allele was not associated with any protective DRB1-DQB1 haplotypes. Of note, however, the association of DPB1*02:01, DPB1*03:01, and DPB1*04:02 with Type 1A diabetes was relatively weak in Japanese children, as the association was significant in the case-control study but not in the TDT. However, DPB1*09:01 was identified as a protective allele in both the case-control study and the TDT,

and DPB1*09:01 appeared to be associated with the protective DRB1*15:02-DQB1*06:01 haplotype, which is a major protective haplotype in the Japanese population but is rare in Caucasian populations.

The independent effects of HLA-A and B have been demonstrated in Caucasian populations (2, 4, 5). Following adjustment for LD to haplotypes at the DR-DQ region, both susceptible and protective alleles were found at HLA-A (e.g., A*24:02, susceptible allele; A*11:01, protective allele) and HLA-B (e.g., B*39:06, susceptible allele; B*57:01, protective allele) (4, 5). A*24:02 was a susceptible allele independent of the susceptible DRB1-DQB1 haplotypes among Japanese children with Type 1A diabetes. A*11:01 was also a protective allele among Japanese children with Type 1A diabetes. However, the association of A*24:02 and A*11:01 with Type 1A diabetes was relatively weak in the Japanese children, as the association was significant in the case-control study but not in the TDT (Tables 3 and 4). Of note, the B*39:06 and B*57:01 alleles were not observed in this study.

The analysis of LD between DRB1-DQB1 haplotypes and DPB1, A, C, or B alleles demonstrated both susceptible (C*08:01-B*40:06-DRB1*09:01-DQB1*03:03 and C*01:02-B*54:01-DRB1*04:05-DQB1*04:01) as well as protective (C*12:02-B*52:01-DRB1*15:02-DQB1*06:01-DPB1*09:01 and A*33:03-C*14:03-B*44:03) haplotypes among Japanese children with Type 1A diabetes.

In terms of genomic imprinting of the HLA-class II gene, several studies have been reported (26–29). In a Caucasian population, a striking feature of the data was that HLA-DR3/DR4 patients inherit their DR3 allele from their mother and the DR4 allele from their father more often than vice versa. Margaritte-Jeannin et al. (27) proposed that parental imprinting for a specific allelic combination may explain the HLA genotypes observed in the patients and their relatives. Sadauskaite-Kuehne et al. (28) also studied diabetes-associated allelic transmission rates from mothers and fathers to children with diabetes in 125 families in Lithuania, an area with a low incidence of type 1 diabetes. They reported that the DR4-DQB1*03:02-DQA1*03:01 haplotype was transmitted significantly more frequently from both parents, but that the DR3-DQB1*02:01-DQA1*05:01 haplotype was transmitted more frequently from only mothers. In Japan, Sasaki et al. (29) reported that maternal alleles in a susceptible DQA1*03:01-DQB1*03:02 haplotype showed a strong transmission disequilibrium with GADAb-positive type 1 diabetes, while paternal alleles in the same haplotype did not in 28 nuclear families, supporting the hypothesis that an epigenetic mechanism including genomic imprinting at the HLA-DQ region is involved in the pathogenesis and the genetic complexity of Japanese type 1 diabetes. However, none of the DRB1, DQB1, DPB1, A, C, or B alleles were preferentially transmitted from the mother or the father to the children with Type 1A diabetes in this study (Table 3). Our study suggests that the genomic imprinting of HLA-class II and class I genes is not involved in the pathogenesis of Type 1A diabetes in Japanese patients.

The frequency of subjects with two susceptible DRB1-DQB1 haplotypes was significantly higher among the children with Type 1A diabetes than among their parents. Of note, the frequencies of homozygosity for DRB1*09:01-DQB1*03:03 and of heterozygosity for DRB1*04:05-DQB1*04:01 and DRB1*08:02-DQB1*03:02 were significantly higher among children with Type IA diabetes, while the frequency of subjects with one susceptible haplotype and without a protective allele (group III) was not different between children with Type 1A diabetes and their parents. The frequencies of subjects with one susceptible haplotype and a protective allele (group IV) and with no susceptible haplotype and a protective allele (group VI) were lower among the children with Type 1A diabetes than among their parents (Table 5). These results suggest a dose effect of susceptible DRB1-DQB1 haplotypes and the effect of protective alleles.

The siblings of children with Type 1A diabetes may also represent a high-risk group for type 1 diabetes in the Japanese population, as the high prevalence (about 4%) of diabetes among Japanese siblings is comparable with that among Caucasian siblings (about 6%) (7, 8). The prevalences of the susceptible DRB1 and DQB1 alleles were similar between the children with Type 1A diabetes and their siblings. However, the prevalence of the protective DQB1*06:01 allele was higher among non-diabetic siblings. The frequency of group IV (no susceptible haplotype and a protective allele) was higher among the siblings than among the children with Type 1A diabetes. These results suggest the role of the protective allele among the siblings.

Only the allele frequency of DRB1*09:01 was significantly different among four age groups of Japanese children with Type IA diabetes determined according to the age at the time of onset (0–1, 2–5, 6–9, and 10–16 years). DRB1*09:01 may be strongly associated with an early onset in preschool children, whereas DRB1*08:02 may be weakly associated with a later onset in school-age children. Murao et al. (15) focused on the differences in the contributions of HLA-DR and -DQ haplotypes to the susceptibility to Type 1 diabetes during adulthood (later than 20 years of age) and childhood (1.0–18 years of age) in Japanese patients. They reported that the DRB1*09:01-DQB1*03:03 (DR9) frequency/DRB1*04:05-DQB1*04:01 (DR4) frequency increased with an increasing age of onset, and that another susceptible haplotype, DRB1*08:02-DQB1*03:02 (DR8), was involved only in the childhood-onset group. They did not mention any

difference among childhood-onset type 1 diabetes, and our results complement the data reported by Murao et al. The present results are also compatible with and complementary to our previous report, in which the frequency of the DR9 genotype was found to be significantly higher among a younger age group (0–10 years) than among an older age group (11–16 years) at the time of onset, and the frequency of DR4-DQ4 was higher in the older age group (11–16 years) (13).

Kawabata et al. (30) reported the age-related association of the MHC class I chain-related gene A and a marker in the class I C region with Japanese type 1 diabetes. However, this study did not show an association of susceptible class I A*24:02, C*01:02, C*08:01, or B*54:01 alleles with age at the time of onset in children with Type 1A diabetes (data not shown).

The amino acid residue at position 57 of the DQβ chain has been shown to play a key role in genetic susceptibility to type 1 diabetes. The lack of aspartic acid at this position at both DQ alleles is strongly associated with type 1 diabetes in Caucasian populations (31, 32). However, this Asp57 hypothesis is not tenable for Japanese type 1 diabetic patients (33). The influence of the HLA-DR and HLA-DQ molecules on the risk of type 1 diabetes is probably related to their central role in antigen presentation and the activation of a helper T cell-mediated immune response (2, 32). The HLA-class II and class I pocket structure is critical to the etiology of autoimmunity, as different pocket variants may have different affinities to the antigenic peptides of specific proteins from pancreatic β cells, including insulin and GAD; therefore, certain variants are more likely to present autoantigenic peptides to T cells than others (32, 34). In a future study, an analysis of how variations in amino acids, especially those found within the peptide-binding domains, are correlated with changes in disease risk would be valuable, providing a possible link between genetic association studies and the causal mechanism(s) of Type 1A diabetes.

In conclusion, this study demonstrated the characteristic association, which was mostly different but partly the same as that in Caucasian populations, of HLA-DRB1, DQB1, DPB1, and A, C, B, genes with Type 1A diabetes among Japanese children. A TDT did not reveal the genomic imprinting of HLA-class II and class I genes in Type 1A diabetes in the present population. A comparison of children with Type 1A diabetes and their parents and siblings suggested a dose effect of susceptible DRB1-DQB1 haplotypes and the effect of protective alleles on the immunological pathogenesis of Type 1A diabetes. These results may provide fundamental data for further genetic studies examining other immune-related and insulin resistance or beta cell function-related genes in Japanese patients with type 1 diabetes.

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Conflict of interest

No potential conflicts of interest relevant to this article were present.

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ORIGINAL ARTICLE

Pro198Leu missense polymorphism of the glutathione peroxidase 1 gene might be a common genetic predisposition of distal symmetric polyneuropathy and macrovascular disease in Japanese type 2 diabetic patients

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ABSTRACT

Aims/Introduction: We have previously reported that the Pro198Leu missense polymorphism in the glutathione peroxidase 1 (GPx-1) gene was associated with frequent macrovascular disease (MVD). Our goal was to examine whether the GPx-1 genotype is associated with diabetic neuropathy.

Materials and Methods: We determined the GPx-1 genotype in 173 Japanese type 2 diabetic patients who received medical interviews, physical examinations, nerve conduction studies, quantitative vibratory perception (QVP), head-up tilt and heart rate variability tests by polymerase chain reaction-restriction fragment-length polymorphism. Diabetic sensorimotor distal symmetric polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN) were evaluated separately. DSPN and DAN were defined by two or more abnormalities of neuropathic leg symptoms, diminished Achilles tendon reflexes or impaired QVP in toes, and two autonomic dysfunctions, respectively. The association of the GPx-1 genotype with DSPN, DAN, MVD and other clinical manifestations was analyzed.

Results: The prevalence of DSPN, impaired QVP and painful leg cramps in patients having a genotype with Pro/Leu at the codon 198 (Pro/Leu type) was significantly higher than those with Pro/Pro type. As a result of multivariate analyses that contained the GPx-1 genotype as an independent variable, the Pro/Leu type was extracted as a significant risk factor of DSPN, QVP impairment and MVD. The statistical significance did not disappear, even after proteinuria, retinopathy and a history of MVD were introduced as independent variables. In contrast, the GPx-1 genotype was not associated with DAN.

Conclusions: The Pro198Leu missense polymorphism of the GPx-1 gene might have a common genetic predisposition to DSPN and MVD. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00127.x, 2011)

KEY WORDS: Glutathione peroxidase 1 gene, Diabetic distal symmetric polyneuropathy, Macrovascular disease

INTRODUCTION

It is well known that macrovascular diseases (MVD), such as myocardial and cerebral infarction, are more common in patients with impaired glucose tolerance[1,2]. Recently, a higher prevalence of polyneuropathy in patients with impaired glucose tolerance, as compared with healthy subjects, has been reported[3]. The similarities in risk factors of diabetic polyneuropathy and MVD have also been reported[4,5]. These findings suggest that there might be a common underlying etiological mechanism in both complications.

One of the most plausible common etiological factors of these complications is the excess of oxidative stress. Elevated reactive oxygen species produced by hyperglycemia induces an oxidation of low-density lipoprotein, and an induction of monocyte chemoattractant protein 1 and adhesion molecules. These mechanisms seem to be mainly implicated in the development of MVD[6]. Excessive oxidative stress is also implicated in the development of diabetic neuropathy[7]. Thus, oxidative stress and the related molecular derangements are widely thought to be a common underlying cause of diabetic complications[8]. We have previously reported that the Pro198Leu missense polymorphism of the glutathione peroxidase 1 (GPx-1) gene is associated with a reduction in transcription and enzyme activity of GPx-1, which is an important anti-oxidative enzyme[9]. Furthermore, we and other investigators have reported significant associations

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between the polymorphism of the GPx-1 gene and MVD[9,10]. A recent study reported a significant association between a GPx-1 gene variant, which was deferent from our study, and peripheral neuropathy in Caucasian subjects[11]. However, to date, there has been no study to show the association between the GPx-1 gene polymorphism and diabetic neuropathy in Japanese patients. In the present study, we aimed to examine whether Pro198Leu polymorphism in the GPx-1 gene is associated with not only MVD, but also diabetic neuropathy.

Diabetic neuropathy is roughly classified into three types: (i) chronic sensorimotor distal symmetric polyneuropathy (DSPN); (ii) diabetic autonomic neuropathy (DAN); and (iii) focal and multifocal neuropathies, according to a statement by the American Diabetes Association[12]. Although DSPN and DAN are specific complications of diabetes, focal and multifocal neuropathies are not. As we have observed that exacerbating factors of sensory and autonomic functions were different[13], subtypes of diabetic neuropathy, DSPN and DAN were separately evaluated. Additionally, various quantitative neurological functions, such as vibratory perception thresholds, nerve conduction parameters and autonomic functions, were also individually evaluated. Then associations between the GPx-1 gene polymorphism and these subtypes of diabetic neuropathy or quantitative neurological functions were investigated.

MATERIALS AND METHODS

Study Design and Participants

A total of 173 unrelated Japanese type 2 diabetic patients (54 outpatients and 119 hospitalized patients of Wakayama Medical University Hospital) who received serial neurological examinations and agreed to be involved in the genetic study were enrolled after giving written informed consent. The study was approved by the ethics committee of Wakayama Medical University and carried out in accordance with the Helsinki Declaration (revised in 2000). In order to evaluate neurological functions accurately, aged patients (more than 70 years) and patients with severe liver or renal dysfunction, cerebrovascular disease with residual neurological deficits, peripheral arterial disease (second degree of Fontaine classification or more) or other neurological diseases were excluded. Diabetes was diagnosed according to the criteria set by the World Health Organization. Hypertension was defined by a blood pressure > 130/80 mmHg or receiving antihypertensive treatment. Patients with total cholesterol > 5.17 mmol/L (200 mg/dL) and/or triglycerides > 1.70 mmol/L (150 mg/dL) and/or high-density lipoprotein cholesterol < 1.03 mmol/L (40 mg/dL) or those on antihyperlipidemic medication were defined as dyslipidemic. MVD was defined by a previous history of cardiovascular disease and/or stroke without residual neurological deficits. Fair and poor glycemic controls were defined as HbA_{1c} (%) < 8.4% and HbA_{1c} (%) ≥ 8.4%, respectively. The value for HbA_{1c} (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[14].

Genotyping of GPx-1

Genomic DNA was isolated from peripheral blood according to standard procedures. The GPx-1 genotype for the Pro198Leu missense polymorphism in exon 2 was analyzed by polymerase chain reaction-restriction fragment-length polymorphism using *HaeIII* as a restriction enzyme, as previously described[9]. The genotypes with Pro/Leu and Pro/Pro at the codon 198 are described as Pro/Leu type and Pro/Pro type, respectively.

Assessment of Neurological Functions

To evaluate the clinical diabetic neuropathy, the sensorimotor and autonomic symptoms were evaluated. Subjective symptoms were ascertained using five criteria: 'numbness in toe and sole'; 'pain in feet, particularly below the knee: pain in feet'; 'painful leg cramp occurring two or more times in a month: painful leg cramp'; 'dizziness on standing: orthostatic dizziness'; and 'frequent constipation/diarrhea or their alternation: frequent constipation/diarrhea'. In the present study, numbness means an uncomfortable sensation with or without ordinary stimulation and dullness in perception inclusively. Painful muscle cramp was defined as a spasm of the calf muscle with severe pain. Achilles tendon reflex (ATR) in the knee-standing position was also examined bilaterally. Furthermore, four objective and quantitative tests were carried out to assess the sensory, motor and autonomic nerve functions as previously described[15]. All examinations were carried out in a temperature-controlled room at 25°C.

Quantitative Vibratory Perception Threshold

Quantitative vibratory perception threshold (QVP) at 125 Hz was assessed using a vibratory sensation meter (AU-02A; RION Company, Tokyo, Japan), whose output level could be changed from −10 to 40 dB (0 dB ref 0–3 m/s^2)[16]. First, the patient put the plantar aspect of their big toe on a vibrating plate and was shown the vibration output level from minimum to maximum. Then the patient was asked to respond by saying 'buzzing' when they felt vibration during a gradual increase of vibratory stimulation. When the patient responded at the same output level twice or more, we regarded that as the perceptible threshold. Measurements were carried out bilaterally and an average of the two sides was used for analysis.

Autonomic Nerve Function Tests (Head-up Tilt Test and Heart Rate Variability Test)

Sympathetic vasomotor function was evaluated by a head-up tilt test using a tilt table (Sakai, Tokyo, Japan) and an automatic sphygmomanometer (BP-88; Colin Company, Tokyo, Japan). Orthostasis-induced decreases in systolic blood pressure after passive standing for 5 min in a 70° head-up position (ΔBP) were examined.

Parasympathetic cardiovagal function was also evaluated by a heart rate variability test. Coefficients of variation of R-R intervals on electrocardiogram after 15 min resting in the supine position (CVR-R) were determined with an electrocardiograph (Autocardiner FCP-2201; Fukuda Denshi, Tokyo, Japan).

Nerve Conduction Study

Motor nerve conduction velocity (MCV) between the wrist and elbow, compound muscle action potential (CMAP) of the ulnar nerve, sensory nerve velocity (SCV) between the wrist and elbow, and sensory nerve action potential (SNAP) of the median nerve were measured bilaterally using standard methods with an electromyograph (Synax 1200; NEC, Tokyo, Japan). Electric stimuli were produced at supramaximal intensity. The CMAP and SNAP produced by the wrist stimulation were evaluated. Skin temperature was measured at the forearms and was maintained at 32°C.

Decision of Abnormality and Subtypes of Diabetic Neuropathy

QVP, MCV, CAMP, SCV, SNAP and logarithmic CVR-R were distributed normally, values exceeding the range of means ± 2 SD of the age-matched healthy subjects in our institution were judged as impaired. Abnormal ΔBP was defined by the American Autonomic Society criteria[17]. Namely, a fall in systolic blood pressure of more than 20 mmHg and/or a fall in diastolic blood pressure of more than 10 mmHg was judged to be an abnormal value. We then classified various neurological manifestations into two subtypes of diabetic neuropathy, distal symmetric polyneuropathy (DSPN) and diabetic autonomic neuropathy (DAN). DSPN was defined by two or more abnormalities in specific neuropathic leg symptoms (numbness in toes and soles, and/or pain in feet), bilaterally diminished ATR and impaired QVP. Painful leg cramp was not included as a neuropathic symptom. For example, this symptom is recognized as a sign of a circulatory disturbance in the questionnaire of Michigan Neuropathy Screening Instruments (MNSI) announced from the website of the Michigan Diabetes Research and Training Center. Neurological Symptom Score (NSC) in the Mayo Clinic also does not contain painful muscle cramp as a sensory symptom[18]. DAN was diagnosed by the two autonomic dysfunctions, impaired CVR-R and abnormal ΔBP.

Statistical Analysis

All statistical analyses were carried out with the StatView program for Windows (version 5.01; SAS Institute, Cary, NC, USA). Differences of clinical data, neuropathic symptoms, ATR, various nerve function data and subtypes of diabetic neuropathy between the two diabetic groups divided based on the GPx-1 genotype were analyzed by ANOVA and χ^2-test.

Multiple logistic regression analyses were carried out to verify the associations between clinical manifestations of diabetic neuropathy and clinical background factors, including the GPx-1 genotype. DSPN, DAN and painful leg cramps were set as dependent variables for the analyses. Eight clinical background factors (age, sex, duration of diabetes, hypertension, dyslipidemia, glycemic control, body mass index [BMI] and GPx-1 genotype: Pro/Pro = 0, Pro/Leu = 1) were selected as independent variables (model 1). Additional analyses, which added proteinuria and retinopathy as independent variables, were also carried out (model 2). In order to negate the influence of MVD on diabetic neuropathy, another analysis was carried out (model 3) in which the history of MVD was added as an independent variable of model 2. An association between MVD and clinical background factors was also evaluated by the two analyses (model 1 and 2).

Multiple regression analyses were also used to determine independent associations between the GPx-1 genotype and six actual results of nerve function tests using the same three sets of independent variables (model 1, 2, 3). A P-value of <0.05 was considered statistically significant.

RESULTS

GPX-1 Genotype

Genotype frequencies (%) of Pro/Pro type, Pro/Leu type and Leu/Leu type were 86.1, 13.9 and 0 in all diabetic patients, respectively. Genotype distributions did not significantly differ from Hardy–Weinberg equilibrium expectations. The frequency of Pro/Leu type in diabetic patients with DSPN was significantly higher than that in the patients without DSPN (17/79 = 21.5% vs 7/94 = 7.5%, P = 0.0076). The frequencies of Pro/Leu type in diabetic patients with DAN was not significantly different from those in patients without DAN (3/25 = 12.0% vs 21/148 = 14.2%, P = 0.7696).

Relationships Between GPx-1 Genotype and Clinical, Neurological Data

Patients were divided into two groups (Pro/Pro type and Pro/Leu type) based on the codon 198 polymorphism, and clinical and neurological features were then compared. Clinical characteristics of the two diabetic groups, such as age, sex, duration of diabetes, therapy, BMI, hypertension, dyslipidemia, recent HbA_{1c}, proteinuria, retinopathy and history of MVD are shown in Table 1. Though the prevalence of MVD tended to be higher in Pro/Leu type than Pro/Pro type (P = 00510), there was no significant difference in clinical characteristics.

The data of subjective symptoms, ATR, subtypes of diabetic neuropathy and quantitative nerve functions are also shown in Table 1. As a subjective symptom, the prevalence of painful leg cramps in Pro/Leu type was significantly higher than that in Pro/Pro type. Among the two subtypes of diabetic neuropathy, only DSPN showed a significantly higher prevalence in Pro/Leu type compared with Pro/Pro type (70.8 vs 41.6, P = 0.0076). In the quantitative neurological data, statistically significant differences between Pro/Leu type and Pro/Pro type were observed in QVP with a prevalence of impaired QVP. In contrast, there was no significant difference in the autonomic or nerve conduction functions between Pro/Leu and Pro/Pro type.

Table 1 | Comparison of clinical characteristics and neurological functions between two diabetic groups divided based on GPx-1 genotype (n = 173)

	Pro/Leu type	Pro/Pro type	P-value
n	24	149	
Clinical characteristics			
Age (year)	55.6 ± 13.0	54.9 ± 10.3	0.8503
Gender (Male/Female)	13/11	85/64	0.7916
Duration of diabetes (years)	12.9 ± 7.8	11.3 ± 7.7	0.3433
Therapy (insulin/OHA/diet and exercise)	1/5/18 (4.2/20.8/75.0)	6/42/101 (4.0/28.2/67.8)	0.7524
BMI (kg/m^2)	23.1 ± 4.2	23.9 ± 3.9	0.3163
Hypertension	10/24 (41.7)	67/149 (45.0)	0.7627
Dyslipidemia	10/24 (41.7)	72/149 (48.3)	0.5445
HbA_{1c} (%)	9.83 ± 2.22	9.11 ± 2.06	0.1134
Proteinuria (no/intermittent/persistent)	16/4/4 (66.6/16.7/16.7)	102/19/28 (68.5/12.7/18.8)	0.8614
Retinopathy (no/simple/pre-, proliferative)	10/4/10 (41.7/16.6/41.7)	78/22/49 (52.4/14.7/32.9)	0.6124
History of macrovascular disease (MVD)	5/24 (20.8)	12/149 (8.1)	0.0510
Subjective symptoms and Achilles tendon reflex (ATR)			
Numbness in toes and soles	9/24 (37.5)	52/149 (34.9)	0.8045
Pain in feet	3/24 (12.5)	16/149 (10.7)	0.7978
Painful leg cramp	14/24 (58.3)	36/149 (24.2)	**0.0006**
Orthostatic dizziness	4/24 (16.7)	27/149 (18.4)	0.8411
Frequent constipation/diarrhea	1/24 (4.2)	13/149 (8.7)	0.4429
Diminished ATRs	19/24 (79.2)	94/149 (64.8)	0.1669
Subtypes of diabetic neuropathy and quantitative nerve functions			
DSPN (Distal symmetric polyneuropathy)	17/24 (70.8)	62/149 (41.6)	**0.0076**
DAN (diabetic autonomic neuropathy)	3/24 (12.5)	22/149 (14.8)	0.7696
QVP (dB)	26.0 ± 7.2	20.4 ± 10.3	**0.0114**
Prevalence of impaired QVP	17/24 (70.8)	56/149 (37.6)	**0.0022**
CVR-R (%)	1.98 ± 0.92	1.96 ± 1.06	0.9591
Prevalence of impaired CVR-R	13/24 (56.5)	68/149 (46.9)	0.3908
ΔBP (mmHg)	7.79 ± 12.49	11.02 ± 14.51	0.3057
Orthostatic hypotension	4/24 (16.7)	35/149 (23.5)	0.4579
MCV (m/s)	50.9 ± 3.9	50.4 ± 6.8	0.7506
Prevalence of impaired MCV	5/24 (20.8)	50/149 (33.6)	0.2141
CMAP (mV)	7.12 ± 3.36	7.10 ± 2.74	0.9734
Prevalence of impaired CMAP	5/24 (20.8)	16/149 (10.7)	0.1599
SCV (m/s)	56.4 ± 5.2	57.3 ± 5.9	0.5187
Prevalence of impaired SCV	10/24 (41.7)	58/149 (38.9)	0.7987
SNAP (μV)	18.4 ± 14.9	21.1 ± 14.3	0.3993
Prevalence of impaired SNAP	8/24 (33.3)	39/149 (26.2)	0.4644

Numbers in parenthesis indicate the percentage. OHA, oral hypoglycemic agents; BMI, body mass index; ATR, Achilles tendon reflex; QVP, quantitative vibratory perception thresholds; CVR-R, correlation coefficient of R-R intervals in electrocardiogram; BP, blood pressure; CMAP, compound muscle action potential; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential; Pro/Leu type, genotype with Pro/Leu at the codon 198 of glutathione peroxidase 1 gene; Pro/Pro type, genotype with Pro/Pro at the codon 198 of glutathione peroxidase 1 gene. The value for HbA_{1c} (%) was estimated as an NGSP equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%. Statistically significant P-value was shown by boldfaced type.

Multivariate Analyses

Relationships between the GPx-1 genotype and a subtype of diabetic neuropathy, painful leg cramp and MVD were analyzed by multiple logistic regression analyses and are shown in Table 2. Multiple logistic regression analysis showed that the GPx-1 genotype (Pro/Leu type) was a significant risk factor for DSPN, painful leg cramps and MVD independent from age, sex, duration, hypertension, dyslipidemia, recent glycemic control and BMI (model 1). The GPx-1 genotype (Pro/Leu type) was a significant risk factor for DSPN, painful leg cramps and MVD, even if proteinuria and retinopathy were added as independent variables of the analyses (model 2). In contrast, Pro/Leu type was not identified as a significant risk factor for DAN in the two regression models.

Associations between the GPx-1 genotype and quantitative neurological functions analyzed by multiple regression analyses are shown in Table 3. Though the GPx-1 genotype (Pro/Leu type) was identified as a significant exacerbation factor of QVP independent from age, sex, duration, hypertension, dyslipidemia, recent glycemic control and BMI, no significant relationship between the GPx-1 genotype and other autonomic or nerve

Table 2 | Relationships between the glutathione peroxidase 1 gene polymorphism and subtype of diabetic neuropathy, painful leg cramp, macrovascular disease evaluated by multiple logistic regression analysis

Independent variables	Model 1 dependent variables				Model 2 dependent variables			
	Subtypes of diabetic neuropathy		Painful leg cramp	History of MVD	Subtypes of diabetic neuropathy		Painful leg cramp	History of MVD
	DSPN	DAN			DSPN	DAN		
R^2 (*P*-value)	R^2 = 0.122 (*P* = 0.0003)	R^2 = 0.137 (*P* < 0.0001)	R^2 = 0.086 (*P* = 0.0217)	R^2 = 0.209 (*P* = 0.0031	R^2 = 0.210 (*P* < 0.0001)	R^2 = 0.530 (*P* < 0.0001)	R^2 = 0.102 (*P* = 0.0202)	R^2 = 0.215 (*P* = 0.0078)
	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value
Age (years)	1.010 (0.976–1.044) 0.5783	**1.039 (1.003–1.076) 0.0345**	0.998 (0.963–1.034) 0.9125	**1.118 (1.022–1.224) 0.0152**	1.010 (0.974–1.047) 0.5809	1.001 (0.934–1.072) 0.9806	0.977 (0.961–1.033) 0.8620	**1.122 (1.023–1.231) 0.0151**
Sex (female: 0, male: 1)	1.054 (0.534–2.077) 0.8800	1.098 (0.552–2.186) 0.7892	0.561 (0.273–1.152) 0.1154	**7.212 (1.609–32.334) 0.0098**	1.099 (0.518–2.329) 0.8059	1.883 (0.458–7.743) 0.3803	0.488 (0.229–1.037) 0.0621	**6.342 (1.375–29.265) 0.0179**
Duration (years) (≧5: 0. 6–15: 1, 16≦: 2)	**1.997 (1.252–3.188) 0.0037**	**2.003 (1.254–3.200) 0.0037**	0.981 (0.598–1.609) 0.9395	1.123 (0.518–2.434) 0.7684	1.343 (0.795–2.270) 0.2706	0.978 (0.409–2.336) 0.9596	1.133 (0.650–1.976) 0.6587	1.031 (0.255–2.335) 0.9413
Hypertension (no: 0, yes: 1)	**2.477 (1.199–5.119) 0.0143**	1.823 (0.881–3.776) 0.1057	0.774 (0.361–1.660) 0.5104	1.590 (0.510–4.961) 0.4244	2.117 (0.958–4.679) 0.0637	2.595 (0.706–9.535) 0.1508	0.753 (0.339–1.674) 0.4864	1.395 (0.429–4.543) 0.5801
Dyslipidemia (no: 0, yes: 1)	**0.412 (0.205–0.830) 0.0130**	**0.463 (0.231–0.931) 0.0308**	0.573 (0.274–1.197) 0.1384	0.801 (0.254–2.524) 0.7050	**0.465 (0.218–0.989) 0.0467**	0.842 (0.212–3.344) 0.8070	0.510 (0.239–1.088) 0.0816	0.741 (0.229–2.393) 0.6160
Glycemic control (~fair: 0, poor: 1)	1.383 (0.699–2.736) 0.3513	1.664 (0.828–3.344) 0.1526	1.572 (0.756–3.269) 0.2261	0.791 (0.261–3.270) 0.1745	1.728 (0.821–3.638) 0.1496	**9.232 (1.991–43.945) 0.0046**	1.410 (0.669–2.971) 0.3663	0.728 (0.234–2.265) 0.5832
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	0.845 (0.552–1.294) 0.4387	**0.572 (0.369–0.885) 0.0122**	0.980 (0.626–1.535) 0.9301	1.624 (0.807–3.270) 0.1745	0.863 (0.547–1.363) 0.5285	**0.158 (0.055–0.456) 0.0006**	0.985 (0.627–1.549) 0.9486	1.606 (0.797–3.236) 0.3330
GPx-1 genotype (Pro/Pro : 0, Pro/Leu :1)	**3.390 (1.252–9.181) 0.0163**	0.891 (0.338–2.351) 0.8157	**4.333 (1.718–10.929) 0.0019**	**3.886 (1.078–14.009) 0.0380**	**3.303 (1.175–9.285) 0.0234**	0.340 (0.045–2.571) 0.2957	**4.653 (1.813–11.943) 0.0014**	**3.782 (1.044–13.703) 0.0428**
Proteinuria (no: 0, intermittent: 1, persistent: 2)					0.694 (0.399–1.205) 0.1941	0.664 (0.302–1.459) 0.3078	1.545 (0.879–2.714) 0.1303	1.350 (0.621–2.936) 0.4494
Retinopathy (no: 0, simple: 1, PPDR~: 2)					**2.919 (1.764–4.831) <0.0001**	**39.232 (5.792–265.745) 0.0002**	0.659 (0.388–1.119) 0.1228	1.009 (0.491–2.076) 0.9802

BMI, body mass index; CI, confidence interval; DAN, diabetic autonomic neuropathy; DSPN, distal symmetric polyneuropathy; GPx-1, glutathione peroxidase 1 gene; MVD, macrovascular disease, OR, odds ratio; PPDR, preproliferative diabetic retinopathy; R^2, decision coefficient. Statistically significant *P*-value was shown by boldfaced type.