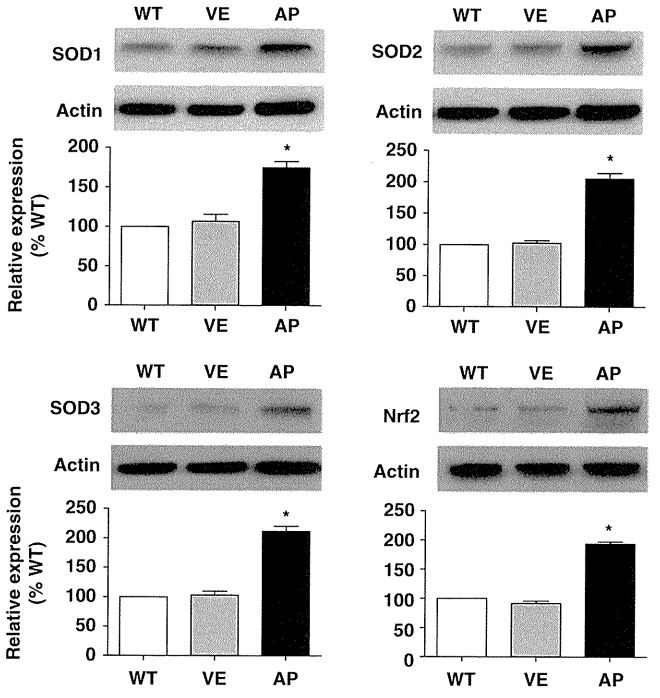

Figure 4 Western blot analysis of renal cortical SOD isoform and Nrf2 expression after an 8-week treatment with the vehicle (VE) or apocynin (AP) in C57BL/6-Akita diabetic mice. WT indicates non-diabetic C57BL/6-wild-type mice. The relative intensity of the SOD-to-actin or Nrf2-to-actin ratios to WT is also shown in the lower panels. Data are presented as the mean ± s.e.m. n=4 per group. $^{*}P<0.001$ *vs.* VE.

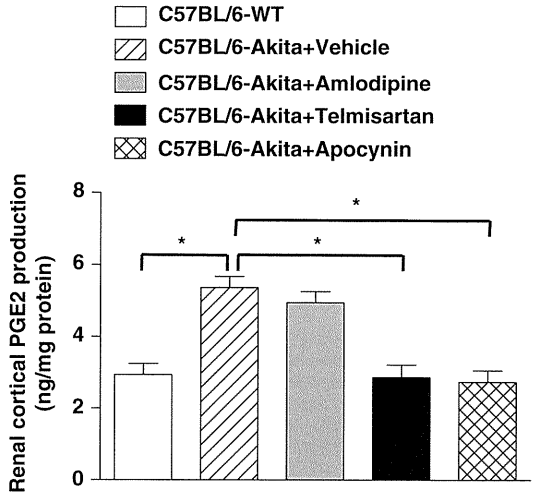

Figure 5 Renal cortical PGE2 levels after a 4-week treatment with the vehicle, amlodipine or telmisartan and after an 8-week treatment with apocynin in C57BL/6-Akita diabetic mice. Data are presented as the mean ± s.e.m. n=5 per group. $^{*}P<0.001$.

Akita diabetic mice. In the SMART study, it was reported that changes in the urinary albumin-to-creatinine ratio from baseline to the end of the treatment period were −36% in type 2 diabetic and microalbuminuric patients treated with valsartan, an ARB, for 24 weeks and +30% in those treated with amlodipine for 24 weeks, despite comparable blood pressure reductions.[38] These results indicate that monotherapy with amlodipine may be insufficient for albumin-to-creatinine ratio reduction. However, the SMART study also suggested that if blood pressure is sufficiently lowered by amlodipine treatment, albumin-to-creatinine ratio would also be reduced to some extent. Similar to the results of the SMART study, this study showed that treatment with telmisartan, not amlodipine, reduced urinary albumin levels and normalized elevated GFR that reflects glomerular hypertension in C57BL/6-Akita diabetic mice, despite comparable levels of hyperglycemia and blood pressure between the two groups treated with these drugs. Further blood pressure reduction with amlodipine treatment may be needed to reduce urinary albumin and glomerular hypertension in C57BL/6-Akita diabetic mice.

In this study, we used the tail cuff method to measure blood pressure. Our data did not indicate a difference in blood pressure between the telmisartan-treated and amlodipine-treated C57BL/6-Akita diabetic mice. However, the tail cuff method has several limitations in blood pressure measurement. Therefore, measuring blood pressure with the telemetry method may be necessary to precisely assess the effects of anti-hypertensive drugs on reducing blood pressure in C57BL/6-Akita diabetic mice.

Our experiments revealed the novel finding that telmisartan not only reduced NAD(P)H oxidase activity but also enhanced SOD activity in the kidneys of C57BL/6-Akita diabetic mice. As expected, renal alterations of these enzymes resulted in a reduction of renal superoxide levels. In contrast, treatment with amlodipine failed to modulate renal NAD(P)H oxidase and SOD enzymes. The differences in renoprotection between telmisartan and amlodipine may in part be attributed to their ability to modulate renal NAD(P)H oxidase and SOD enzymes. A recent experimental study reported that treatment with low doses of telmisartan (0.1–0.3 mg kg^{-1} per day) did not affect renal SOD activity in non-diabetic mice.[25] However, our study indicated that treatment with a high dose of telmisartan (5 mg kg^{-1} per day) enhanced renal SOD activity in C57BL/6-Akita diabetic mice. It is possible that high doses of this ARB are needed to enhance renal SOD activity. Paralleling the elevation of renal SOD activity in telmisartan-treated C57BL/6-Akita diabetic mice, our immunohistochemical study revealed increases in the protein expression of SOD1 and SOD3 isoforms in glomeruli and of the SOD2 isoform in the proximal tubules of these mice. Western blot analysis confirmed the finding that telmisartan therapy increases the protein expression of SOD1, SOD2 and SOD3 in the kidneys of C57BL/6-Akita diabetic mice.

As angiotensin II signaling directly promotes NAD(P)H oxidase activation,[22,23] the downregulation of renal NAD(P)H oxidase by AT1 receptor blockade is a reasonable result. However, the mechanism by which telmisartan upregulates renal SOD remains unclear. We hypothesized that NAD(P)H oxidase would negatively regulate the renal expression of SOD or transcription factor Nrf2, which is known to upregulate several antioxidant enzymes including SOD.[26–28] To test this hypothesis, we treated C57BL/6-Akita diabetic mice with an NAD(P)H oxidase inhibitor, apocynin, for 8 weeks and investigated the alteration of renal SOD and Nrf2 expression. As expected, apocynin treatment markedly lowered renal NAD(P)H oxidase activity in C57BL/6-Akita diabetic mice, resulting in the reduction of renal superoxide levels. The reduction of renal superoxide by apocynin therapy contributed to reducing urinary albumin levels and normalizing the elevated GFR in C57BL/6-Akita diabetic mice. Consistent with the results of recent experimental studies,[29,39] apocynin did not lower the blood pressure of C57BL/6-Akita diabetic mice. Although the cause is unclear, one possible explanation is that unlike telmisartan, apocynin is not effective in blocking systemic vasoconstriction by angiotensin II. More importantly, we found that inhibiting NAD(P)H

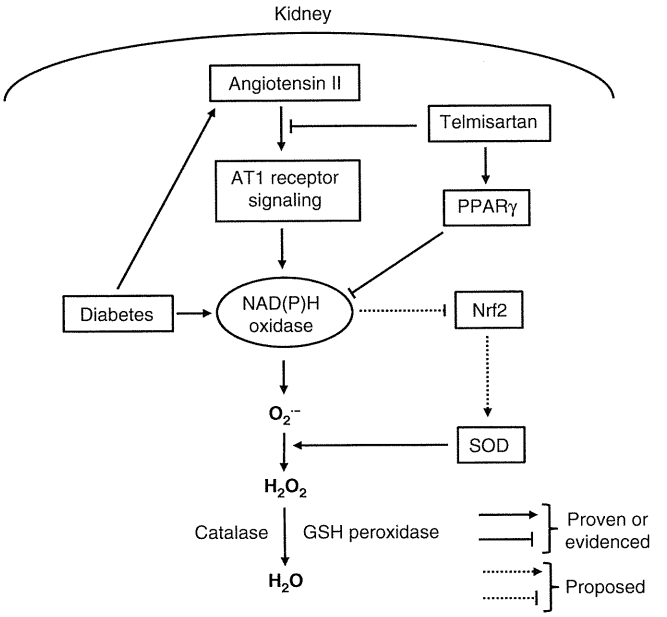

Figure 6 The proposed mechanism by which telmisartan upregulates renal SOD in diabetes.

oxidase upregulated renal SOD and Nrf2 in C57BL/6-Akita diabetic mice. Suppression of NAD(P)H oxidase by telmisartan also resulted in increased expression of renal Nrf2. Taken together, our data suggest that NAD(P)H oxidase negatively regulates renal SOD, possibly by downregulation of Nrf2, and that telmisartan could upregulate renal SOD by the suppression of NAD(P)H oxidase and subsequent upregulation of Nrf2 (Figure 6).

This study does not demonstrate that other members of the ARB class also have the ability to upregulate renal SOD. As AT1 receptor blockade by other members of the ARB class, such as olmesartan, has been shown to reduce renal NAD(P)H oxidase expression,[24] all ARBs may share the renal SOD upregulating effect to some extent. However, as shown in a recent clinical study,[20] there is a difference in the ability to ameliorate oxidative stress among the ARB members. Of the currently available ARBs, telmisartan is known to have the strongest binding affinity to AT1 receptors, the longest half-life, and a high lipophilicity.[40,41] In addition, telmisartan also acts as a partial agonist of peroxisome proliferator-activated receptor-γ. An experimental study of obese and hypertensive rats has indicated that peroxisome proliferator-activated receptor-γ activation by pioglitazone therapy downregulates NAD(P)H oxidase.[42] These properties may explain the more powerful antioxidative and renoprotective effects of telmisartan by modulation of renal NAD(P)H oxidase and SOD.

Reactive oxygen species, including superoxide anions, induce overproduction of vasodilatory PGE2 through cyclooxygenase-2 upregulation.[43] PGE2 is a vasodilator for afferent arterioles, and excessive PGE2 could cause the glomerular hypertension observed in early diabetes.[44,45] Therefore, it is thought that the reduction of renal superoxide with apocynin normalized elevated GFR in C57BL/6-Akita diabetic mice. In addition, renal superoxide reduction with telmisartan is likely to affect glomerular pressure reduction. This study revealed that treatment with apocynin or telmisartan reduces renal PGE2 production in C57BL/6-Akita diabetic mice. Furthermore, the ability of telmisartan to dilate efferent arterioles through AT1 receptor blockade also contributes to the amelioration of glomerular hypertension. Thus, renal superoxide reduction is thought to provide beneficial effects in improving abnormalities in glomerular hemodynamics in early diabetes.

In conclusion, we report a novel finding that AT1 receptor blockade by telmisartan treatment could upregulate renal SOD enzyme by the suppression of NAD(P)H oxidase and subsequent upregulation of Nrf2, leading to an improvement of oxidative stress in kidneys exposed to hyperglycemia. These effects are expected to greatly contribute to the amelioration of earlier diabetic renal change observed in C57BL/6-Akita diabetic mice.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Diabetes, Obesity and Metabolism 2011.

Comparisons of the effects of 12-week administration of miglitol and voglibose on the responses of plasma incretins after a mixed meal in Japanese type 2 diabetic patients

To compare the effects of miglitol [an alpha-glucosidase inhibitor (AGI) absorbed in the intestine] and voglibose (an AGI not absorbed) on plasma glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) levels, 26 and 24 Japanese type 2 diabetic patients were randomly assigned to receive miglitol or voglibose, respectively. After 12-week administration of both drugs, during 2-h meal tolerance test, plasma glucose, serum insulin and total GIP were significantly decreased and active GLP-1 was significantly increased. Miglitol group showed a significantly lower total GIP level than voglibose group. Miglitol, but not voglibose, significantly reduced body weight (BW). In all participants, the relative change in BW was positively correlated with that of insulin significantly and of GIP with a weak tendency, but not of GLP-1. In conclusion, both drugs can enhance postprandial GLP-1 responses and reduce GIP responses. The significant BW reduction by miglitol might be attributable to its strong GIP-reducing efficacy.

Keywords: diabetes, incretin, GIP, GLP-1, miglitol, voglibose

Date submitted 16 June 2011; date of first decision 26 July 2011; date of final acceptance 21 October 2011

Introduction

Novel approaches to the treatment of type 2 diabetes (T2D) have focused on strategies for enhancing the action of glucagon-like peptide-1 (GLP-1), such as the use of GLP-1 mimetics and dipeptidyl peptidase-4 (DPP-4) inhibitors. Gastric inhibitory polypeptide (GIP), another incretin, has also been shown to have a physiological role on fat accumulation into adipose tissues [1]. In patients with obesity and insulin resistance, reducing the GIP signal may be a novel therapeutic strategy to prevent and cure T2D.

In studies with healthy controls, administration of alpha-glucosidase inhibitors (AGIs) leads to a reduction in the postprandial GIP responses and a prolonged enhancement of the GLP-1 responses. However, a few studies with a small number of patients with T2D, including our own, have reported controversial effects of AGIs on GLP-1 (no change following acarbose or voglibose administration, [2–4]; increase following miglitol administration [4–6]) and GIP (no change following voglibose administration [4], decrease following acarbose and miglitol administration [2,4–6]) plasma levels after ingestion of a mixed meal. Furthermore, it is unclear whether these effects of AGIs differ based on the type of AGI used and whether they are preserved after long-term administration.

Miglitol is an AGI absorbed in the upper portion of the small intestine, resulting in the diminished efficacy of AGI at the lower portion of the small intestine. Therefore, in the clinical use of miglitol, a sufficient dosage with strong AGI efficacy can be administered with lower incidence of gastro-intestinal adverse events compared with other AGIs not absorbed in the intestine [7].

Therefore, we attempted to compare the effects of 12 week of treatment with miglitol and voglibose (an AGI not absorbed in the intestine) on the responses of plasma incretins after a mixed meal in Japanese patients with T2D.

Patients and Methods

This 12-week, multi-center, open, randomized parallel controlled study included 50 Japanese patients with T2D, who were treated with diet therapy alone or with oral hypoglycaemic agents other than AGIs. All patients were instructed to continue their usual diet and medications through the study.

Before and after the 12-week administration of miglitol or voglibose (50 mg of miglitol or 0.3 mg of voglibose thrice a day immediately before every meal), all patients underwent a 2-h meal tolerance test (MTT) performed in the morning after an overnight fast; the meal consisted of 460 kcal of total caloric load with 56.5 g of carbohydrates, 18 g of protein and 18 g of fat.

During the MTT, blood samples were collected at 0, 30, 60 and 120 min. For determination of the plasma levels of active GLP-1 and total GIP, tubes (Mitsubishi Chemical Medience Co., Tokyo, Japan) containing EDTA-2Na and Diprotin A (a DPP-4 inhibitor) were used. Active GLP-1 and total GIP levels were measured using commercially available ELISA kits (Millipore Corporation, Billerica, MA, USA). Active GLP-1 was

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measured using plasma prepared with solid phase extraction (Oasis HLB Extraction Plate, Waters Corporation, Milford, MA, USA). The value for haemoglobin A1c (HbA1c) (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA1c (NGSP) (%) = HbA1c as defined by the Japan Diabetes Society (%) + 0.4%.

As there are no reports focusing on differences in the specific chronic effects of different types of AGIs on circulating incretin levels in humans, we could not predefine an appropriate sample size. Therefore, this study should be considered as exploratory research.

The study protocol was approved by the ethics committee of each participating institute. All participants provided their written informed consent. This study has been registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry System as trial ID 000001671.

Results

Clinical characteristics of the 50 patients (26 miglitol and 24 voglibose recipients) are shown in the Table 1. Both drugs significantly decreased HbA1c levels, whereas the fasting plasma glucose (PG), insulin and serum lipid levels were not affected. Only miglitol decreased body weight (BW) and body mass index significantly.

PG, insulin, total GIP and active GLP-1 responses during the MTT (figure 1) at baseline were comparable in both groups. Following the administration of both drugs, PG and insulin levels measured at 30 and 60 min during the MTT were significantly lower than those measured at baseline, except for the insulin levels measured at 30 min in the voglibose group. During the second MTT, PG levels at 30 min were significantly lower in the miglitol group than in the voglibose group. In the miglitol group, PG levels at 120 min during the second MTT were higher than those at 60 min, whereas in the voglibose group, PG levels at 120 min were lower than those at 60 min.

Postprandial total GIP levels during the second MTT were significantly lower than baseline in both groups, with a lower overall level observed in the miglitol group compared with the voglibose group (Table 1 and figure 1C). The active GLP-1 area under the curve (AUC) was higher compared with baseline after the administration of both drugs (Table 1). Active GLP-1 at 60 min was higher after administration of both drugs, whereas

Table 1. Clinical characteristics of participants and relative changes from baseline values of the integrated responses of plasma glucose (PG), serum insulin, total gastric inhibitory polypeptide (GIP) and active glucagon-like peptide-1 (GLP-1) during a 2-h meal tolerance test.

	Miglitol		Voglibose	
	Baseline	12 weeks	Baseline	12 weeks
Male/Female, n	14/12		18/6	
Age (years)	58.5 ± 9.9		59.5 ± 11.6	
Duration (years)	9.9 ± 6.7		9.1 ± 6.6	
Use of OADs, n (%)	21 (80.8)		20 (83.3)	
Use of SU, n (%)	17 (65.4)		15 (62.5)	
Use of MET, n (%)	16 (61.5)		17 (70.8)	
Use of TZD, n (%)	9 (34.6)		11 (45.8)	
Body weight (kg)	64.5 ± 14.0	63.6 ± 14.0**	69.4 ± 18.7	69.3 ± 18.6
BMI (kg/m^2)	25.1 ± 5.2	24.7 ± 5.1*	25.6 ± 5.0	25.6 ± 4.9
FPG (mmol/l)	8.4 ± 1.3	8.3 ± 1.3	8.8 ± 2.7	8.2 ± 1.8
Fasting insulin (pmol/l)	30.0 ± 22.6	27.5 ± 19.7	29.9 ± 31.1	31.9 ± 40.5
TC (mmol/l)	5.40 ± 0.61	5.60 ± 0.70	5.17 ± 0.73	5.40 ± 0.98
TG (mmol/l)	1.48 ± 0.71	1.55 ± 0.98	1.39 ± 0.46	1.49 ± 0.99
HDLc (mmol/l)	1.51 ± 0.42	1.55 ± 0.53	1.52 ± 0.40	1.48 ± 0.35
HbA1c (%)	7.51 ± 0.47	7.35 ± 0.61*	7.49 ± 0.70	7.13 ± 0.71*
Δ from baseline (%)		−0.16 ± 0.39		−0.36 ± 0.39
PG AUC (h.mmol/l)	23.9 ± 0.81	19.5 ± 0.57***	23.4 ± 0.69	20.2 ± 0.76***
Relative changes (%)		−17.0 ± 2.5$		−12.8 ± 2.0
Insulin AUC (h.pmol/l)	210.5 ± 24.5	149.5 ± 20.0***	214.5 ± 45.0	182.0 ± 42.0**
Relative changes (%)		−24.9 ± 5.6		−10.6 ± 5.8
GIP AUC (h.pg/ml)	752.4 ± 54.2	385.5 ± 31.0***,†	813.5 ± 71.6	489.6 ± 33.9***
Relative changes (%)		−47.9 ± 2.3‡		−35.4 ± 4.6
GLP-1 AUC (h.pmol/l)	8.3 ± 0.84	11.4 ± 0.92***	8.5 ± 0.74	10.1 ± 0.98*
Relative changes (%)		+52.0 ± 10.3‡		+33.0 ± 14.2

Data are expressed as mean ± SD unless otherwise indicated. BMI, body mass index; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDLc, high-density lipoprotein cholesterol; OADs, oral anti diabetic agents; SU, sulfonylurea; MET, metformin; TZD, thiazolidinedione; Δ from baseline (%), changes from baseline values; Relative changes (%), relative changes (%) of the respective parameters at 12 weeks from baseline values; AUC, area under the curve during the 2-h meal tolerance test.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. baseline values revealed by the Wilcoxon signed-ranks test, respectively.

†$p < 0.05$ vs. voglibose treatment revealed by the Mann–Whitney U-test.

‡$p < 0.01$ and $ $p < 0.001$ vs. voglibose treatment revealed by the analysis of covariance (ANCOVA) model with the respective baseline values as covariates.

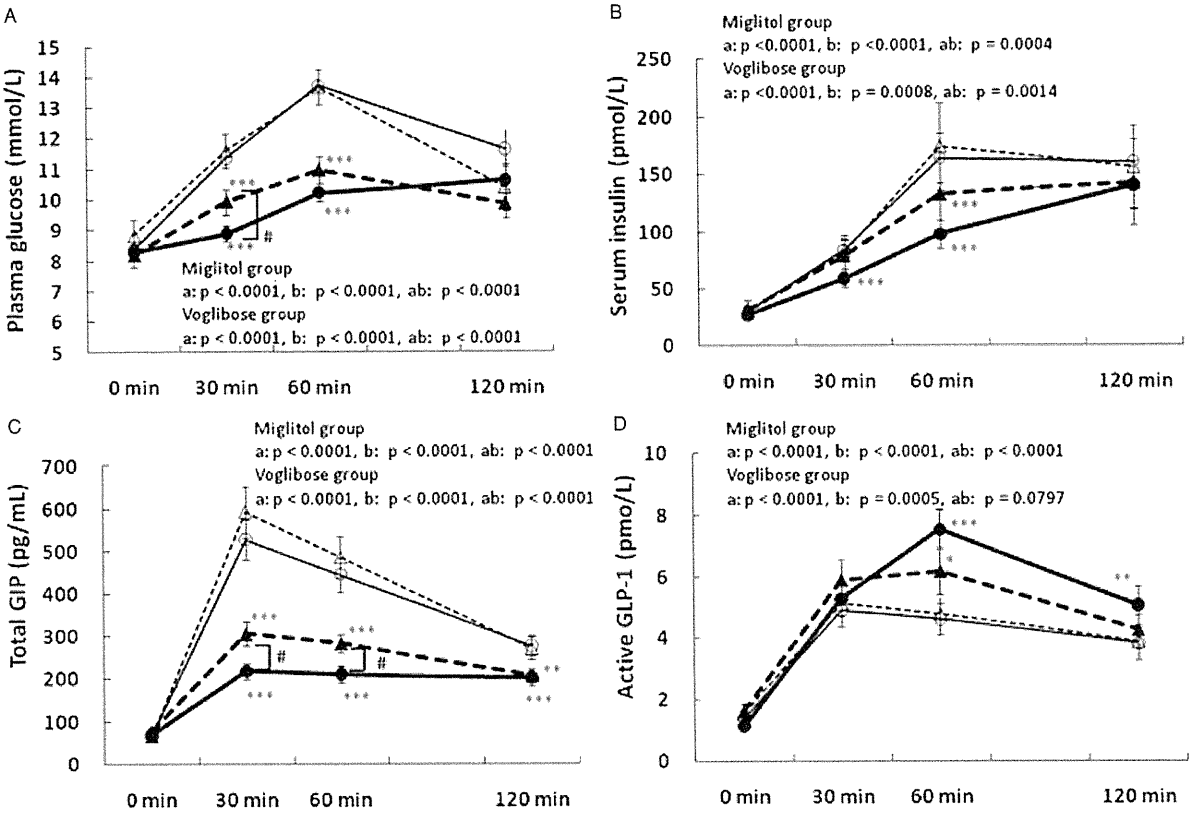

Figure 1. The effects of 12-week administration of miglitol and voglibose on changes in plasma glucose (A), serum insulin (B), plasma total gastric inhibitory polypeptide (GIP) (C) and plasma active glucagon-like peptide-1 (GLP-1) (D) in response to ingestion of a mixed meal. Values are expressed as mean ± SE. Open circle, before miglitol; closed circle, after miglitol; open triangle, before voglibose; closed triangle, after voglibose. p-Values represent differences according to treatment (a), over time course (b), and the interaction of treatment and time course (ab) as calculated by repeated-measures analysis of variance. *, ** and ***, significant differences ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) at individual time points by the Wilcoxon-signed ranks test. #, $p < 0.05$ between miglitol and voglibose by the Mann–Whitney *U*-test.

active GLP-1 at 120 min was higher only after administration of miglitol, compared with baseline (Figure 1D).

An analysis of covariance (ANCOVA) with the respective baseline values as covariates revealed that the relative changes (%) from baseline in the PG AUC, total GIP AUC and active GLP-1 AUC at 12 weeks were significantly different between miglitol and voglibose (Table 1).

Pearson's correlation analyses were performed to assess the relationships between the relative changes from baseline (%) in the AUCs of plasma incretins and those in the PG AUC, the insulin AUC and the ratio of incremental AUC from 0 min during the MTT of insulin to that of PG (iAUC-insulin/iAUC-PG). Among all participants, the relative change in the total GIP AUC was correlated with that in the insulin AUC ($r = +0.3702$, $p < 0.01$), whereas the relative change in the active GLP-1 AUC was correlated with that in the iAUC-insulin/iAUC-PG ($r = +0.2990$, $p < 0.05$).

The relative change in BW was positively and significantly correlated with the change in the insulin AUC ($r = +0.320$, $p < 0.05$) and also showed a weak correlation with the change in the GIP AUC ($r = +0.262$, $p = 0.066$) among all participants. The relative change in BW was positively correlated with the HbA1c change from baseline in the miglitol group ($r = +0.4121$, $p < 0.05$) but not in the voglibose group ($r = -0.3181$, $p = 0.1298$).

Discussion

To our knowledge, this study is the first to show that relatively long-term (12-week) administration of both miglitol and voglibose significantly increases the responses of active GLP-1 and decreases the responses of total GIP after ingestion of a mixed meal in patients with T2D.

In this study, miglitol showed a lower level of postprandial total GIP and a higher level of active GLP-1 in ANCOVA comparisons with voglibose. The results may have affected the difference in postprandial PG levels between miglitol and voglibose. One of the limitations of this study is the lack of a placebo arm. Therefore, it is necessary to wait for future, more statistically robust investigations before concluding that miglitol has a stronger effect on circulating incretin levels than voglibose.

The relative change in the total GIP AUC was correlated with that in the insulin AUC, with both parameters being reduced in this study, suggesting the effective suppression of carbohydrates absorption in the upper intestine by the two drugs. However,

direct causality between the two hormones is unclear as the insulinotropic effect of GIP is almost abolished in patients with T2D [8]. In contrast, the relative change in the active GLP-1 AUC correlated with that in the iAUC-insulin/iAUC-PG, suggesting improvement of beta cell function through the increased active GLP-1 AUC.

During the second MTT, PG at 30 min during MTT was significantly lower in the miglitol group than in the voglibose group, and the peak PG was delayed from 60 to 120 min after miglitol administration but not after voglibose administration (figure 1), indicating that miglitol, an AGI absorbed in the intestine, induces a relatively lower degree of carbohydrate absorption in the GIP-secreting upper portion of the intestine, and a higher degree of carbohydrate absorption in the GLP-1-secreting lower portion of the intestine than voglibose, which is not absorbed in the intestine. This may also explain the present result, namely the significantly lower level of total GIP and the relatively higher level of active GLP-1 observed in the miglitol group compared with the voglibose group.

In previous studies among patients with T2D, miglitol and acarbose suppressed postprandial GIP responses regardless of AGI dosage or duration of administration [2,4–6]. In contrast, a single and low-dose (0.3, 0.5 and 1.0 mg) administration of voglibose did not suppress either of postprandial PG, insulin or GIP responses [4,9], whereas a prolonged administration of low dose voglibose did suppress these responses, both in healthy subjects (0.5 and 1.0 mg) [9] and in our patients with T2D (0.3 mg), indicating the necessity of a prolonged administration of low-dose voglibose for significant efficacy.

No enhancement of GLP-1 response by acarbose has previously been found among obese patients with poorly controlled T2D (HbA1c, 7.8% [2]; 8.22% [3]), but such an enhancement was revealed among patients with T2D who had relatively good glycaemic control (HbA1c, 6.7–7.0% [4–6]) and who were administered miglitol. Therefore, the positive enhancement of the effects of GLP-1 responses induced by miglitol and voglibose in this study may be partially attributed to the relatively good glycaemic control among our patients (HbA1c, approximately 7.5%). Furthermore, obesity attenuates the GLP-1 response to carbohydrates [10], and none of our patients was severely obese; this may partially explain our present result of the significant enhancement of the postprandial GLP-1 response after administration of both drugs.

The mechanism behind the increase in postprandial active GLP-1 levels after the administration of both drugs is probably attributable to enhanced secretion of GLP-1 and/or inhibition of DPP-4 activity. Because postprandial total GLP-1 levels (measured with antisera against carboxy-terminus of GLP-1) increased after the administration of AGIs in previous studies, including our own [6,11], suggesting increased GLP-1 secretion, we did not measure total GLP-1 levels but measured only active GLP-1 levels, which directly induce insulin secretion and other effects of GLP-1. Interestingly, a recent study in mice reported that voglibose administration decreased DPP-4 activity through a reduction in the DPP-4 protein level despite increases in the gut GLP-1 content and both total and active plasma GLP-1 levels [12]. Accordingly, simultaneous measurements of both GLP-1 and GIP in its active and total forms, as well as DPP-4 activity and its protein levels before and after AGIs usage is warranted in future studies.

We used the highest dose of voglibose (0.3 mg thrice a day) but not the highest dose of miglitol (50 mg and not 75 mg thrice a day) permitted in Japan because a previous crossover study reported that only a single 50 mg dose of miglitol can modulate PG, active GLP-1 and total GIP levels but not a single 0.3 mg dose of voglibose [4]. It remains to be elucidated whether the highest dose of miglitol (75 mg thrice a day) would have a stronger effect on modulating postprandial circulating incretin levels than the dose used in this study.

It is also unclear whether miglitol or voglibose themselves have the potential to increase active GLP-1 levels. In one study in mice [12], DPP-4 activity was not influenced by the addition of voglibose to the plasma. Furthermore, in a study of healthy human subjects [11], plasma GLP-1 levels did not change during ingestion of a carbohydrate-free meal with acarbose. These results suggest that AGIs enhance circulating GLP-1 levels only after their administration with carbohydrates.

In this study, the reduction in BW was positively correlated with that in GIP with a weak tendency and there was a positive correlation between the reductions of HbA1c and BW only in the miglitol group. The postprandial GIP response was significantly lowered after miglitol administration than after voglibose administration. In animal experiments of excess nutrient intake, reducing the GIP signal in a physiological level results in improvement of obesity, insulin resistance and glucose tolerance accompanied with prevention of fat accumulation into adipocytes and high energy expenditure, [1]. Taken together, these findings suggest that sufficient suppression of the GIP signal by miglitol is a preferable therapeutic option for obese patients with T2D.

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Conflict of Interest

The authors have no conflict of interest to declare.

T. N. and H. Y. contributed to design, conduct/data collection, analysis and writing of the manuscript. R. Y.

conducted this study and collected the data. T. S., M. H., T. M. and H. F. conducted this study, carried out data collection and analysed the manuscript. K. T. carried out the analysis. Y. Y. contributed to design, analysis and writing of the 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 $11.9 \pm 2.6\%$ 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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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

		Type 1A		Control		Type 1A vs. Control		
HLA		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.

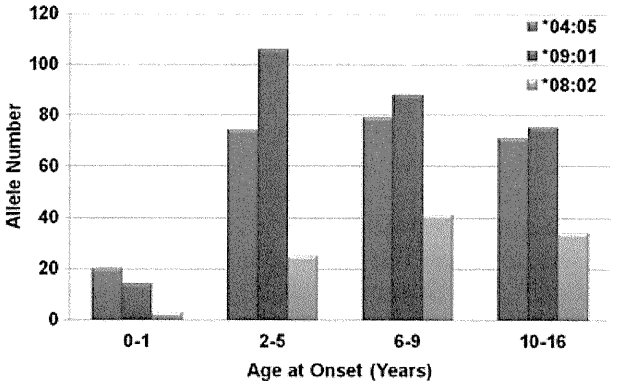

Fig. 1. DRB1 allele frequencies in four age groups of Japanese children with Type 1A diabetes according to the age at onset. The frequency of DRB1*09:01 was higher (Pc < 0.01) in the 2–5-year onset group, while the frequency of DRB1*08:02 tended to be higher in the 6–16-year onset group, although the 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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