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Figure Legends

Figure 1: Systolic blood pressure (SBP, (a)), heart rate (HR, (b)), double product (DP (c)), and shock index (SI (d)) values and their 95% confidence intervals for each week of gestational age, calculated on the basis of a mixed linear model.

Figure 2: Systolic blood pressure (SBP, (a)), heart rate (HR, (b)), double product (DP (c)), and shock index (SI (d)) values and their 95% confidence intervals for each week for a year, calculated on the basis of a mixed linear model without adjusting for seasonal variation.

Figure 3: Systolic blood pressure (SBP, (a)), heart rate (HR, (b)), double product (DP (c)), and shock index (SI (d)) values for the combination of gestational age and expected date of birth, calculated on the basis of a mixed linear model. The horizontal axis shows gestational age, and the vertical axis shows the expected date of birth.

Table 1. Associations between hemodynamic parameters and daily minimum outside temperature in summer and in other seasons.

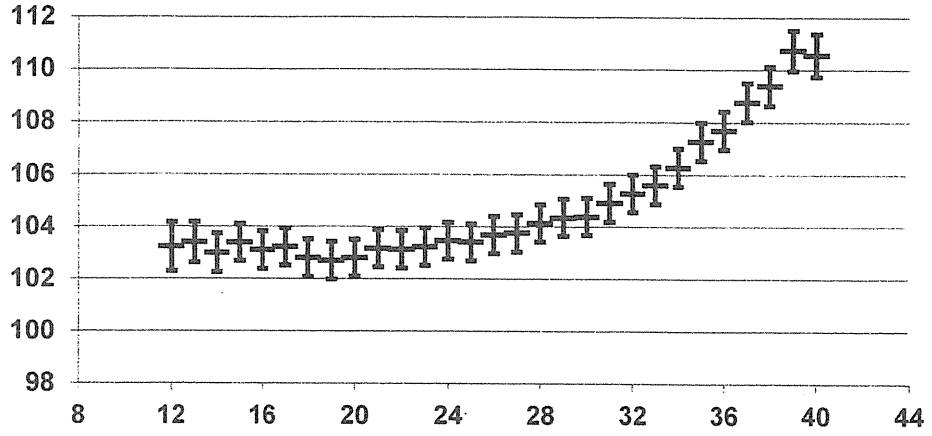
	Summer (from June to September)			Other seasons (from October to May)			Interaction*
	β	SE	p	β	SE	p	p
SBP (mmHg)	–0.3055	0.0049	<0.0001	–0.1999	0.0074	<0.0001	<0.0001
HR (bpm)	0.0095	0.0052	<0.0001	–0.0560	0.0080	<0.0001	<0.0001
DP (10^2·mmHg·bpm)	-0.2172	0.0069	<0.0001	–0.2051	0.0104	<0.0001	<0.0001
SI (10^{-2}·bpm/mmHg)	0.2235	0.0058	<0.0001	0.0811	0.0088	<0.0001	<0.0001

SBP: systolic blood pressure, HR: heart rate, DP: double product, SI: shock index.

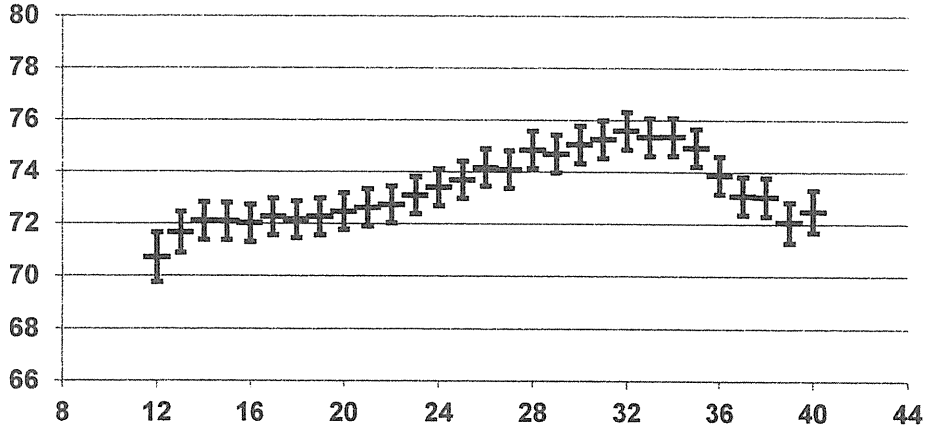
*: Interaction between daily minimum outside temperature and seasonality and hemodynamic parameters.

Figure 1

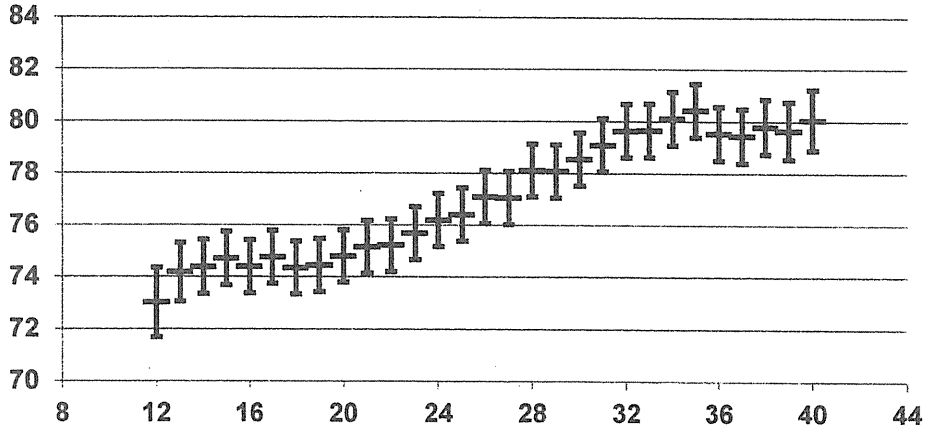
a) SBP (mmHg)



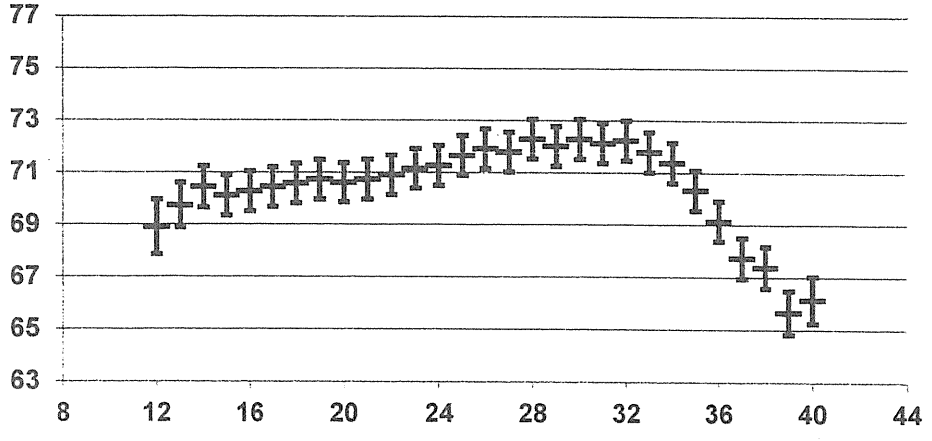
b) HR (bpm)



c) DP(10^2·mmHg·bpm)



d) SI(10^{-2}·bpm/mmHg)



Gestational Age (weeks)

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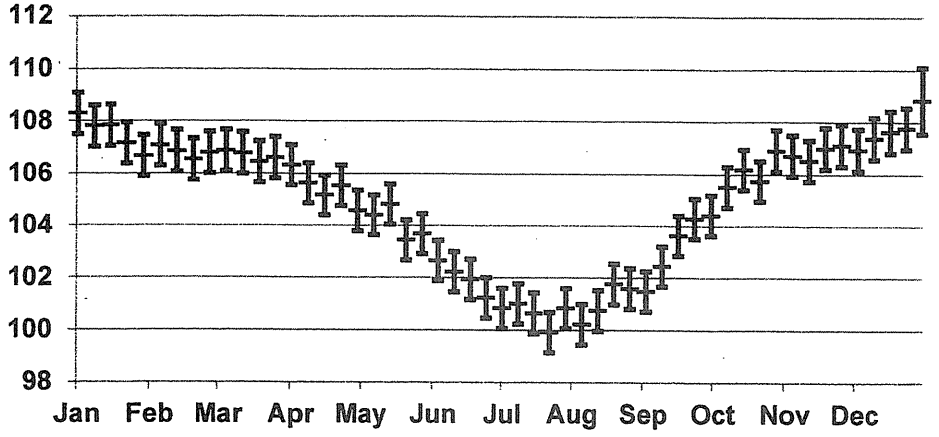
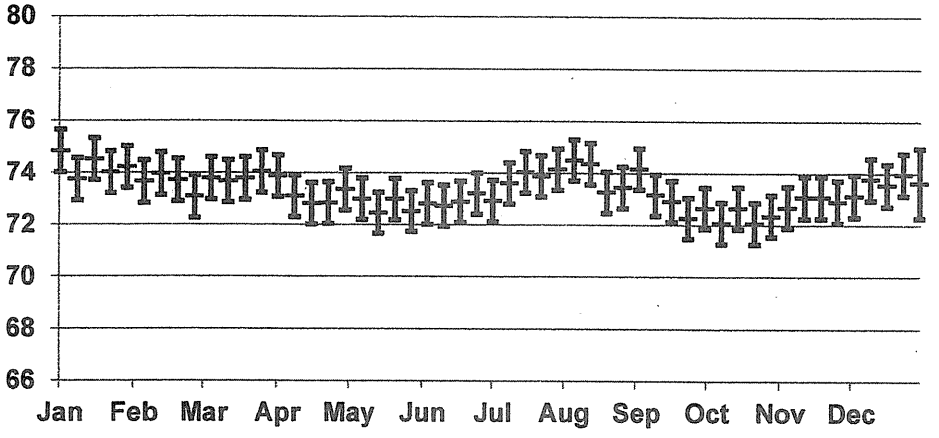
Figure 2

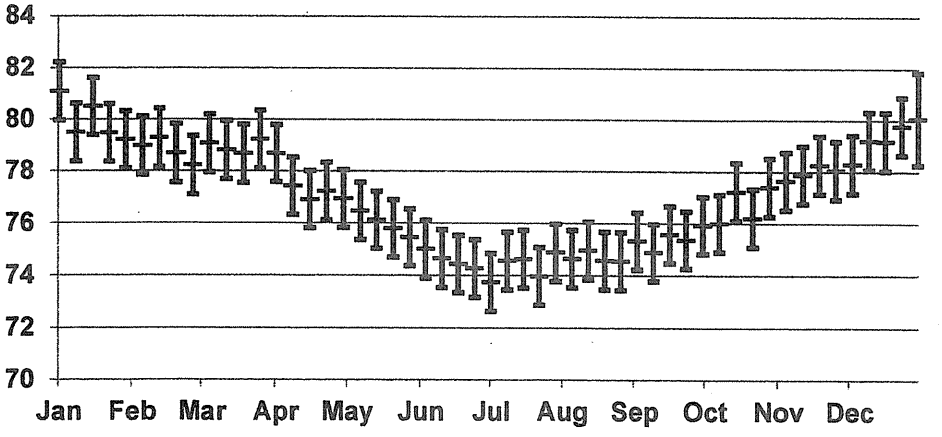
a) SBP (mmHg)



b) HR (bpm)



c) DP(10^2•mmHg•bpm)



d) SI(10^{-2}•bpm/mmHg)

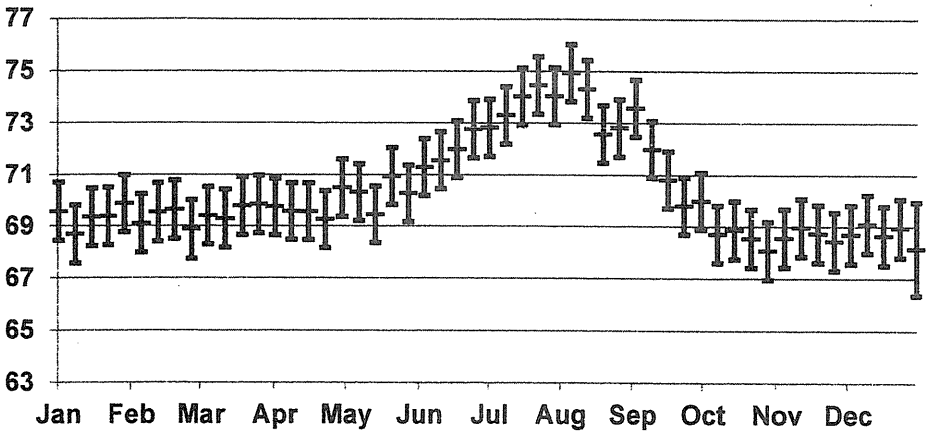


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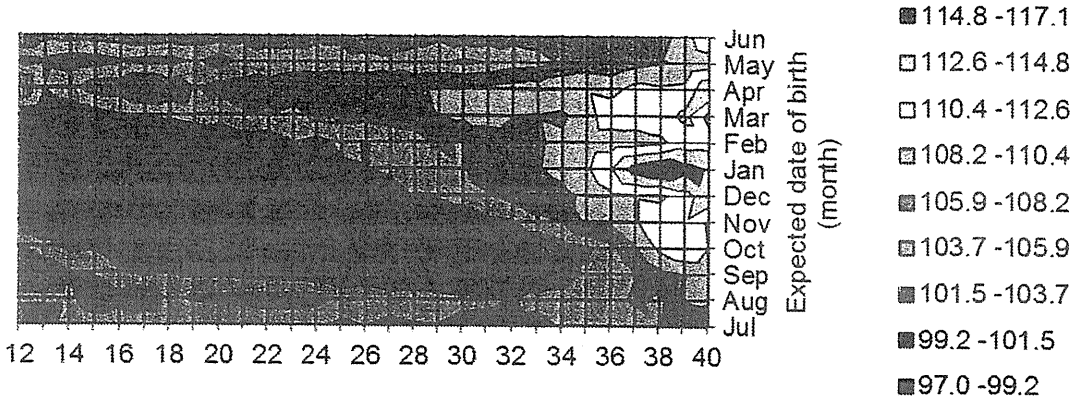
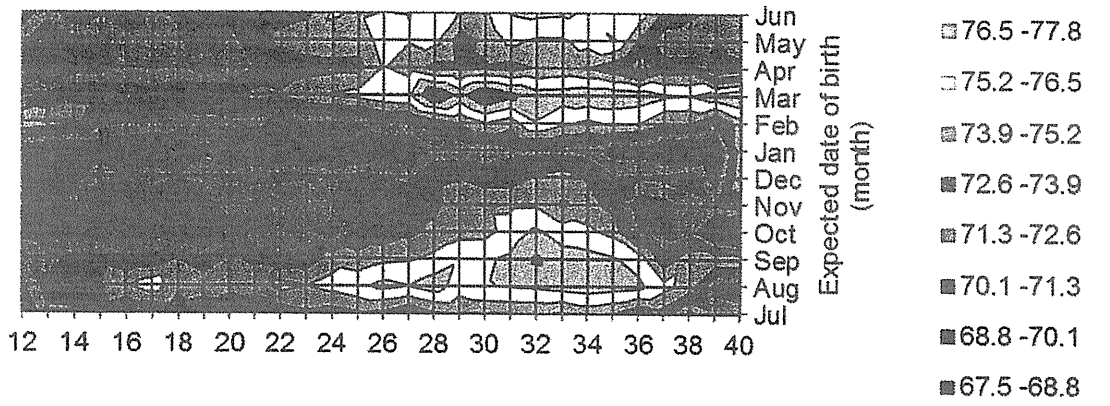
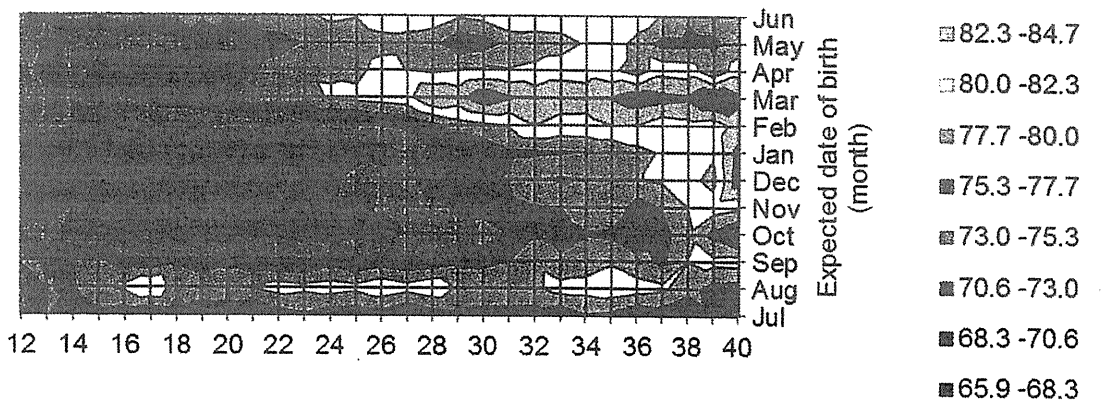
Figure 3
a) SBP (mmHg)



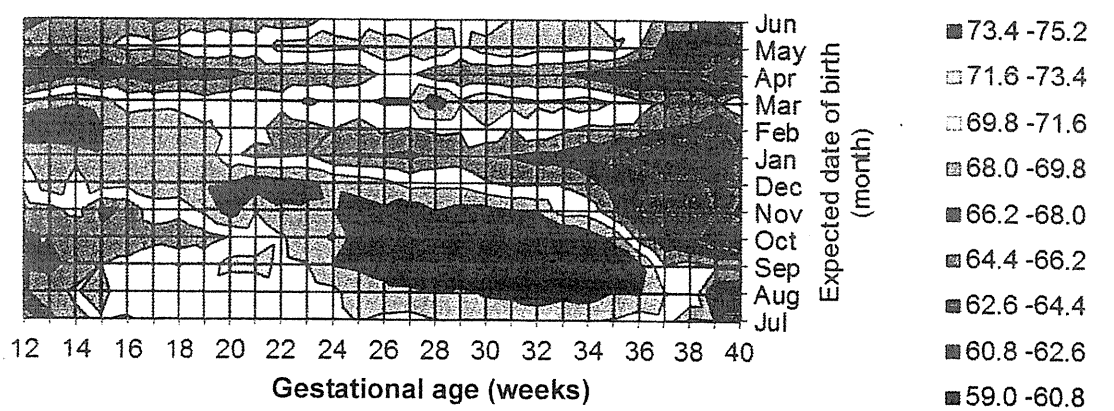
b) HR (bpm)



c) DP(10^2•mmHg•bpm)



d) SI(10^{-2}•bpm/mmHg)



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JCEM ONLINE: BRIEF REPORTS

Identification and Functional Analysis of Novel Human Growth Hormone Secretagogue Receptor (*GHSR*) Gene Mutations in Japanese Subjects with Short Stature

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Abstract

Context: Short stature (SS) is a multifactorial developmental condition with a significant genetic component. Recent studies have revealed that rare deleterious mutations in the GH-secretagogue receptor type 1A (*GHSR1A*) gene could be a cause of familial SS or GH deficiency.

Objective: The aim of this study was to evaluate the contribution of *GHSR1A* mutations to the molecular mechanism underlying SS in Japanese subjects.

Methods: We performed mutational screening of the *GHSR1A* gene in 127 unrelated Japanese SS patients diagnosed with either isolated GH deficiency or idiopathic SS. Identified mutations were analyzed in 188 control subjects, and their functional properties were examined in a heterologous expression system.

Results: Four novel heterozygous *GHSR1A* mutations were identified (ΔQ36, P108L, C173R, and D246A). Expression studies demonstrated that these mutations had varying functional consequences: 1) all mutations showed a loss-of-function effect on the constitutive signaling activity of GHSR1A, but the degree of loss varied widely; 2) C173R caused intracellular retention of the

mutated protein, resulting in total loss of receptor function; 3) P108L resulted in a large decrease in binding affinity to ghrelin, without affecting its surface expression; 4) D246A uniquely impaired agonist- and inverse agonist-stimulated receptor signaling; and 5) ΔQ36 showed only a subtle reduction in constitutive activity. The cumulative frequency of these putative functional mutations was significantly higher in the patient group than in controls (4.72 *vs.* 0.53%; $P = 0.019$; odds ratio = 9.28; 95% confidence interval, 1.10–78.0).

Conclusions: Our results suggest that *GHSR1A* mutations contribute to the genetic etiology of SS in the Japanese population.

Ghrelin exerts pleiotropic effects, including stimulation of GH secretion and enhancement of appetite, through binding and activation of the G protein-coupled GH-secretagogue receptor (GHSR) (1, 2). Two GHSR isoforms have been identified (3, 4); the primary GHSR1A product contains seven-transmembrane domains, whereas GHSR1B is an inactive form with five-transmembrane domains. In view of the ghrelin/GHSR pathway contributing to pituitary GH release, *GHSR1A* is a biological candidate for influencing/modulating height. However, recent genome-wide association studies (5, 6) as well as studies using selected haplotype-tagging single nucleotide polymorphisms (7, 8) did not provide evidence for association between common *GHSR1A* variants and adult or childhood height.

On the other hand, rare but functionally significant *GHSR1A* mutations were discovered in patients with familial short stature (SS) (9–11), thus shedding new light on the physiological importance of the ghrelin/GHSR system in somatic growth. Initially, two missense mutations, A204E and F279L, were identified in an obese patient and a SS child, respectively (9). A204E was subsequently found in two unrelated pedigrees with familial SS, showing a codominant mode of inheritance with incomplete penetrance and variable phenotypic expressivity (10). Functional characterization demonstrated that both mutant receptors had diminished or significantly reduced constitutive activities (CA), although they showed preserved ability to respond to ghrelin (10, 12) [a high constitutive ligand-independent signaling activity, up to ~50% of ligand-stimulated signaling, has been proven for GHSR1A in an *in vitro* setting (13, 14)]. More recently, the first case of compound heterozygosity for W2X and R237W was identified in an SS patient with partial GH deficiency (GHD) (11). In this case, transmission of the GHD phenotype suggested a recessive mode of inheritance. Expression studies showed that the nonsense W2X mutation favored complete loss-of-function, whereas R237W caused only a partial, but potentially important, loss of CA.

In this study, to facilitate elucidation of the molecular etiology of familial/genetic SS, we screened for *GHSR* mutations in a cohort of Japanese patients (n = 127) with isolated GHD or idiopathic SS.

Patients and Methods

All methods are described in more detail in the Supplemental Data (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Subjects

This study was approved by the Ethics Committee for Human Genome/Gene Research of the University of Tokushima. A total of 127 unrelated Japanese individuals, diagnosed with either isolated GHD (n = 14) or idiopathic SS (n = 113) according to established clinical criteria (10, 15), were recruited by the Japan Growth Genome Consortium, a research network of Japanese pediatric endocrinologists. Written informed consent was obtained from all participants. DNA from unrelated healthy Japanese individuals (n = 188) was used as the control.

Mutational analysis

The two *GHSR1A* coding exons were screened for mutations by sequencing (Supplemental Table 1). Frequencies of variant alleles in control subjects were determined by PCR-restriction fragment length polymorphism (Supplemental Table 2).

Transfection studies

Human *GHSR1A* cDNA was used to create either N-terminal hemagglutinin (HA)-tagged or C-terminal enhanced green fluorescent protein (EGFP)-tagged expression constructs (Supplemental Table 1). Mutations were introduced by site-directed mutagenesis. Receptor-mediated luciferase (*luc*) reporter gene assays were performed on transiently transfected HEK293A cells using either serum-responsive element (SRE)-*luc* or cAMP-responsive element-*luc* reporter (12). Whole-cell receptor binding assays were conducted using ^{125}I-labeled ghrelin. Both cell-surface and total protein expression levels of the HA-tagged receptor were determined by a cell-based ELISA (12). Immunoblotting was performed with a horseradish peroxidase-conjugated anti-HA antibody. For deglycosylation experiments, lysates were treated with endoglycosidase H or protein N-glycosidase F (PNGase F). The subcellular distribution of EGFP-tagged receptors was monitored by fluorescence microscopy. Indirect immunofluorescence was performed with an antibody against the endoplasmic reticulum (ER) marker calnexin.

Statistics

Data are presented as mean ± SD. Statistical significance was analyzed using Student's *t* test and Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Novel *GHSR* mutations

Eight *GHSR* sequence variants were identified (Table 1), which included: 1) four novel variants affecting amino acid residues common to both the 1A and 1B isoforms [ΔQ36 (a 3-bp in-frame deletion), P108L, C173R, and D246A; Supplemental Fig. 1, A and B]; 2) a missense substitution of the 1B-specific residue (A277P); and 3) three silent changes (G57G, L118L, and R159R). Heterozygous ΔQ36 was detected in three patients as well as one control. P108L, C173R, and D246A were rare, being found in only a single patient each, all in a heterozygous condition. A277P was detected in one patient and three controls. According to the SIFT and PolyPhen results, P108L and C173R were predicted as having a potentially damaging effect on protein function (Table 1).

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Table 1.

Summary information on *GHSR* variants detected in Japanese population

On the basis of these observations, we decided to focus our secondary efforts on the four novel *GHSR1A* mutations, ΔQ36, P108L, C173R, and D246A. The pedigrees of the six families carrying one of the selected mutations are shown in Supplemental Fig. 2. Little information was available regarding clinical and auxological variables for the propositi, their parents, and other family members. In the present cohort, the cumulative number of these alleles was six in 127 (4.72%) patients and one in 188 (0.53%) controls [$P = 0.019$ by two-tailed Fisher's exact test; odds ratio = 9.28; 95% confidence interval, 1.10–78.0; population attributable risk = 4.21%; 95% confidence interval, 0.37–8.06].

Functional characterization of *GHSR1A* mutations

Expression constructs encoding wild-type (WT) or mutant GHSR1A were used for transient expression in HEK293A cells and subsequent functional evaluation. In a SRE-*luc* reporter assay, when compared with WT or the two previously characterized mutant receptors [A204E and F279L (10, 12)], novel *GHSR1A* mutations displayed different signaling properties (Fig. 1A): 1) ΔQ36 displayed a partial but significant decrease in CA (67.5%) but showed comparable ghrelin (agonist)-induced activation and [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P(SPA) (inverse agonist)-induced inhibition; 2) P108L showed a significant decrease in CA (32.0%) and a normal response against ghrelin, but had less sensitivity to SPA; 3) C173R was devoid of CA with a complete lack of response to ghrelin; and 4) D246A had reduced but significant CA (58.8%) while displaying a significantly lower response to ghrelin (even at 1 μM) and a nearly normal response to SPA. We obtained essentially the same findings in the cAMP-responsive element-*luc* reporter assay (data not shown).

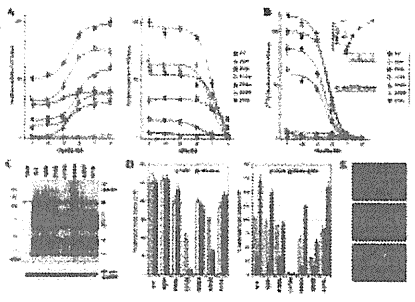

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Fig. 1.

Functional characterization of GHSR1A mutants. A, SRE-*luc* reporter gene assay. HEK293A cells were cotransfected with the WT or a mutant GHSR1A expression construct, pSRE-*luc* and pRL-TK plasmids, and subsequently stimulated with increasing concentrations of ghrelin (agonist; *left*) or SPA (inverse agonist; *right*). The WT receptor (*filled circles*) showed significant basal, ligand-independent CA, and treatment with ghrelin resulted in a concentration-dependent activity increase, whereas SPA inhibited CA. The two previously characterized mutant receptors, A204E (*open triangles*) and F279L (*filled inverted triangles*), showed greatly reduced CA (4.41 and 17.7%, respectively, compared with WT) but retained their ability to respond to ghrelin, these observations being consistent with previous findings (10, 12). The novel GHSR1A mutants displayed different signaling property patterns, as described in the text (ΔQ36, *open circles*; P108L, *filled squares*; C173R, *open squares*; and D246A, *filled triangles*). Transfection of vector alone resulted in no significant *luc* activity (data not shown). Results as compared with WT activity [arbitrarily set at 100, either treated with 1 μM ghrelin (*left*) or not treated (*right*)] are mean ± SD for at least four determinations. B, Whole-cell radioligand binding assay. In HEK293A cells transiently expressing the WT receptor, saturation receptor binding analysis using [^{125}I]ghrelin demonstrated a single class of high affinity, saturable binding sites with Kd of 0.43 nM, comparable to those reported in previous studies (depicted in the *inset*), whereas no specific binding was observed in cells transfected with vector alone (data not shown). Competition binding studies using 100 pM [^{125}I]ghrelin showed high affinity binding of ghrelin (IC_{50} = 7.07 nM) with the WT receptor (*filled circles*), whereas A204E (*open triangles*) displayed no detectable specific binding and F279L (*filled inverted triangles*) showed a small decrease in binding, by approximately 80%, but comparable affinity for ghrelin (IC_{50} = 3.34 nM). Newly identified GHSR1A mutants displayed variable competition binding results as described in the text (ΔQ36, *open circles*; P108L, *filled squares*; C173R, *open squares*; D246A, *filled triangles*). Results as compared with WT binding (arbitrarily set at 100), in the absence of unlabeled ghrelin, are mean ± SD for at least four determinations. C, Immunoblot (IB) analysis. N-terminally HA-tagged WT or mutated GHSR1A was transiently expressed in HEK293A cells, and whole-cell lysates were prepared. Equal protein amounts were resolved in SDS-PAGE, blotted, and probed with anti-HA antibody (*upper panel*). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

expression was evaluated as the loading control (*lower panel*). Size markers (in kilodaltons) are to the *left of the blots*. Results are representative of at least three separate independent transfection experiments yielding similar results. *Arrows* indicate major protein bands of approximately 34, 46, and 62 kDa, and *bracket* indicates a broad high molecular-weight protein band migrating between 60 and 90 kDa. *Filled triangles*, nonspecific band. D, Cell-based ELISA. *Left*, HEK293A cells were transiently transfected with N-terminally HA-tagged WT or mutated GHSR1A expression constructs. The receptor amount was measured by whole-cell ELISA assays, either in permeabilized (for total receptors; *open columns*) or nonpermeabilized (for cell-surface receptors; *black columns*) cells. Data were normalized to the WT receptor expression value and are presented as mean ± SD from three independent experiments, each performed in quadruplicate. *Right*, To evaluate the effects of agonist and inverse agonist treatment on cell-surface receptor expression, at 24 h after transfection, the medium was replaced with serum-free medium (*open columns*) and medium containing either ghrelin (1 μM; *black columns*) or SPA (1 μM; *gray columns*), and the cells were then incubated for an additional 18 h. The cell-surface receptor amounts were quantified by whole-cell ELISA assays. Data were normalized to the WT receptor expression value under the nonstimulated condition and are mean ± SD of three independent experiments, each performed in quadruplicate. Note that ghrelin treatment of WT-expressing cells resulted in significant down-regulation of cell-surface receptor expression (to <40% of that in corresponding nonstimulated cells), whereas, as opposed to ghrelin treatment, surface expression of the WT receptor was significantly increased when cells were exposed to SPA (by approximately 1.7-fold). E, Double immunofluorescent staining. HEK293A cells were transiently transfected with C-terminally EGFP-tagged WT (*top panel*) or mutated GHSR1A construct, either C173R (*middle panel*) or A204E (*lower panel*). The cells were fixed, permeabilized, and processed for indirect immunofluorescent staining with an antibody against calnexin, an ER marker protein. *Green* fluorescence corresponds to EGFP-GHSR1A, and *red* corresponds to calnexin. *Yellow* represents colocalization of green and red. Nuclei stained with DAPI (4′,6-diamino-2-phenylindole) are shown in *blue*.

In whole-cell [^{125}I]ghrelin binding assays, *GHSR1A* mutations displayed variable competition binding results (Fig. 1B): 1) ΔQ36 showed a slight increase in binding (about 1.15-fold) with similar binding affinity to WT (IC_{50} = 4.38 and 7.07 nM for ΔQ36 and WT, respectively); 2) specific binding was virtually undetectable for P108L and C173R; and 3) D246A showed reduced binding (~60%), with comparable binding affinity (IC_{50} = 3.38 nM).

Western blot analysis of WT-expressing cells showed the existence of intense, multiple immunoreactive bands (Fig. 1C). The immunoblot patterns from endoglycosidase H- and PNGase F -digested samples (Supplemental Fig. 3) suggested that: 1) the approximately 34- and 62-kDa bands most likely represent the nonglycosylated monomeric and dimeric forms, respectively; 2) the approximately 46-kDa species is the core-glycosylated monomeric form located in the ER; and 3) the broad band migrating at 60–90 kDa corresponds to the mature, terminally glycosylated, dimeric, or oligomeric forms. The ΔQ36, P108L, D246A, and F279L receptors showed a distribution and intensity of immunoreactive bands essentially similar to WT. In contrast, both C173R and A204E exhibited a selective and profound loss of intensity of 60- to 90-kDa bands, with the former being more severely affected, whereas their approximately 46-kDa species were preserved to some extent (Fig. 1C). These changes appeared to be not associated with an alteration of mRNA levels (*e.g.* decreased transcription, reduced mRNA stability), because the *GHSR1A* transgene levels of transfected cells, as assessed by quantitative RT-PCR, were not significantly different from that of WT-expressing cells (data not shown).

The total protein expression levels of WT and mutant receptors, as assessed by a cell-based ELISA, were largely consistent with immunoblot results (Fig. 1D): 1) total cellular expression of ΔQ36 was equivalent to that of WT; 2) P108L, D246A, and F279L were expressed at a slightly reduced level (60–80%); 3) A204E exhibited significantly lower expression (<60%); and 4) C173R was expressed at the lowest level (<40%). Quantification of cell-surface receptors showed that, with respect to their total expression levels, ΔQ36, P108L, D246A, and F279L were expressed at levels approximately equal to that of WT. In contrast, C173R and A204E displayed either almost complete loss or significantly decreased surface expression (5.4 and 25.6%, respectively, compared with WT). In addition, ghrelin-induced down-regulation of ΔQ36, P108L, and D246A was comparable to that of WT, but was of a lesser degree for C173R and A204E (Fig. 1D). In contrast, the cell-surface expression of F279L did not change in response to ghrelin. The SPA-induced increase in cell surface expression of ΔQ36, D246A, A204E, and F279L, but not P108L and C173R, was comparable with that of WT.

The subcellular distributions of ΔQ36, P108L, D246A, and F279L, as monitored by fluorescence microscopy, were similar to that of WT (Supplemental Fig. 4). In contrast, both C173R and A204E displayed a fine reticular pattern of fluorescence extending from the perinuclear area and distributed throughout the cytoplasm. Double-immunofluorescent staining confirmed that the signals of C173R and A204E overlapped exclusively with that of the ER-marker, calnexin (Fig. 1E).

Discussion

We report herein the identification of four novel *GHSR1A* mutations. Functional characterization, as summarized in Supplemental Table 3, indicates that all the mutations are associated with a loss of CA, thus being consistent with a previous notion that reduced CA is responsible for SS phenotypes (10, 16). On the other hand, we found that the degree of loss could vary greatly, from only modest impairment to complete loss. Our results also highlight that *GHSR1A* mutations can have varying functional characteristics attributable to differences in their mutational mechanisms, *i.e.*: 1) P108L results in a large decrease in binding affinity to ghrelin; 2) C173R likely causes misfolding and aberrant ER retention; and 3) D246A leads to impaired agonist- and inverse agonist-stimulated receptor signaling. Notably, ΔQ36 showed only a subtle reduction in CA, thus raising the possibility that ΔQ36 may be a benign polymorphic variant. However, we cannot rule out the possibility of ΔQ36 having pathological significance, because this situation resembles the case of R237W (11), whose phenotype involves only partial loss of CA. We also provided additional information on previously identified mutations (9, 10, 12), *i.e.* A204E most likely interferes with normal intracellular trafficking resulting in ER retention, but to a much lesser extent than that of C173R, whereas F279L has impaired ability to undergo agonist-mediated receptor down-regulation, a control mechanism determining receptor responsiveness.

It should be mentioned that the *GHSR1A* mutations identified in this study occurred rarely and were each, except for ΔQ36, found only in one patient or a single family, all in a heterozygous condition, and thus their pathological significance in individual patients and families may not be sufficiently elucidated. We found the cumulative frequency of these mutations to be significantly higher in the patient group (4.72 *vs.* 0.53% in controls; $P = 0.019$), supporting that these account for a significant fraction of patients. However, obviously our sample size was small and this preliminary finding also needs to be replicated in a large and genetically homogeneous sample.

In summary, our data emphasize the importance of detailed characterization of the mutational mechanisms and functional consequences of individual *GHSR1A* mutations. Because the ghrelin/GHSR system exerts multiple biological actions in different cell types, the variability in the functional consequences of *GHSR1A* mutations may be associated with variable clinical phenotypes in patients.

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Footnotes

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Abbreviations:

CA	Constitutive activity or activities
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
GHD	GH deficiency
GHSR	GH secretagogue receptor
HA	hemagglutinin
luc	luciferase
SPA	[D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P
SRE	serum-responsive element
SS	short stature
WT	wild-type.

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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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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),