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Class II HLA genotype in fulminant type 1 diabetes: A nationwide survey with reference to glutamic acid decarboxylase antibodies

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ABSTRACT

Aims/Introduction: Fulminant type 1 diabetes is a subtype of type 1 diabetes characterized by a remarkably abrupt onset of insulin-deficient hyperglycemia within a few days. The aim of the present study was to clarify characteristic class II HLA genotypes in a large number of patients with fulminant type 1 diabetes to date.

Materials and Methods: We analyzed the HLA-*DRB1* and *DQB1* genotypes, and their haplotypes in 207 patients with fulminant type 1 diabetes and 325 control subjects in the Japanese population.

Results: The frequencies of the *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* haplotypes were significantly higher, and those of the *DRB1*01:01-DQB1*05:01*, *DRB1*15:02-DQB1*06:01* and *DRB1*08:03-DQB1*06:01* haplotypes were significantly lower in patients with fulminant type 1 diabetes than in the control subjects. Combination analysis showed that the frequencies of homozygotes with *DRB1*04:05-DQB1*04:01* [odds ratio (OR) 7.0] and *DRB1*09:01-DQB1*03:03* (OR 9.5) were significantly higher in patients with fulminant type 1 diabetes. Within a limited portion of patients with fulminant type 1 diabetes with antibodies to glutamic acid decarboxylase (GADab; $n = 25$), the frequency of *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, was significantly higher than in control subjects (44.0% vs 13.7%; $Pc < 0.05$, OR 5.0).

[Correction to last line of Results, added after online publication 29 July 2011: "OR 5.1" is changed to "OR 5.0".]

Conclusions: Our large-scale study showed the characteristic class II HLA genotypes in fulminant type 1 diabetes, and implicated that genetic contribution to disease susceptibility is distinct between GADab-positive and GADab-negative fulminant type 1 diabetes. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00139.x, 2012)

KEY WORDS: Fulminant type 1 diabetes, HLA, Glutamic acid decarboxylase

INTRODUCTION

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes identified in 2000[1–3]. It is defined as diabetes that results from the extremely rapid and almost entire destruction of pancreatic β-cells within a few days. The clinical characteristics of this subtype are different in many aspects from those of typical type 1A diabetes[3]. Although fulminant type 1 diabetes resembles the typical form of type 1 diabetes in that it is characterized by high plasma glucose levels accompanied by ketosis or ketoacidosis, it clearly differs by an extremely acute onset of diabetes, which is confirmed by nearly normal HbA_{1c} levels against high plasma glucose concentration, and virtually no C-peptide secretion at the onset of the disease, indicating that the process of pancreatic β-cell destruction is very rapid.

Fulminant type 1 diabetes is common in the Asian population; it accounts for approximately 20% of ketosis-onset type 1 diabetes in Japan[2,3] and 7% in Korea[4,5]. Furthermore, several cases have been reported from China[6], Taiwan[7], the Philippines[8], Malaysia[9] and France[10].

It is suggested that both genetic factors[11–13] and environmental factors, such as viral infection[14–19], contribute to the pathogenesis of this disease. In regard to genetic factors, it has been reported that class II HLA strongly confers susceptibility to the development of fulminant type 1 diabetes. In the analysis of the serological typing of class II HLA, we have shown that HLA-DR4-DQ4 was significantly more frequent in fulminant type 1 diabetes in Japan[12]. Several studies have so far reported the association of class II HLA genotype with fulminant type 1 diabetes[20–22]; however, the number of patients was limited in these reports as a result of the low incidence of type 1 diabetes in general, fulminant type 1 diabetes in particular, in the Japanese population.

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The aim of the present study was thus to investigate the class II HLA genotypes and re-evaluate the contribution of the class II HLA to susceptibility and resistance to fulminant type 1 diabetes in a large number of patients.

MATERIALS AND METHODS

Subjects and Methods

We examined 207 patients with fulminant type 1 diabetes and 325 healthy control subjects in Japan. Among them, 152 patients with fulminant type 1 diabetes were registered with the committee of the Japan Diabetes Society, and data for the other 55 patients were collected from reports in the literature from June 2000 to March 2007.

Inclusion criteria for fulminant type 1 diabetes were: (i) ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms; (ii) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide <0.3 ng/mL (0.10 nmol/L) or serum C-peptide <0.5 ng/mL (0.17 nmol/L) after glucagon injection or meal load soon after disease onset; and (iii) plasma glucose level ≥16.0 mmol/L (288 mg/dL) and HbA_{1c} <8.9% at the first visit[2]. Healthy control subjects had normal glucose tolerance as assessed by a 75 g oral glucose tolerance test, had no family history of diabetes, and resided in the Ehime and Osaka areas as described previously[23]. GAD antibodies (GADab) were positive in 25 patients and negative in 182 patients (Table 1). We also analyzed 15 patients with pregnancy-associated fulminant type 1 diabetes (PF), 51 female patients of child-bearing age (13–49 years) with fulminant type 1 diabetes that was not associated with pregnancy (NPF) and 70 female control subjects of child-bearing age.

The present study was approved by the ethics committee of the Japan Diabetes Society, and informed consent was obtained from all subjects. The detailed characteristics of these subjects are shown in Table 1.

The value for HbA_{1c} (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[24].

Typing of HLA-DR and -DQ

HLA-DRB1 and *-DQB1* were genotyped by the PCR sequence-specific primer and PCR sequence-specific oligonucleotide methods (Invitrogen, Carlsbad, CA, USA). The most probable *DRB1-DQB1* haplotypes were deduced from known linkage disequilibria.

Statistical Analysis

Clinical data of GADab-negative and -positive fulminant type 1 diabetes was analyzed by using chi-squared-test or Kruskal–Wallis test. Allele frequencies were estimated by direct counting. Genotypes, whose total frequencies in both total subjects with fulminant type 1 diabetes and control subjects were five or more than five, were listed in the present study. The significance of the difference in distribution of alleles between patients with fulminant type 1 diabetes and healthy control subjects was determined by a chi-squared-test. *P*-values were corrected by using the number of different alleles tested (denoted as *Pc*). Statistical significance was defined as $Pc < 0.05$.

RESULTS

Characteristics of GADab-Negative and -Positive Fulminant Type 1 Diabetes

GADab was detected in 25 (12.1%) of 207 patients with fulminant type 1 diabetes in the present study. Therefore, first of all, we compared detailed characteristics between GADab-negative and -positive fulminant type 1 diabetes (Table 1). There were no differences between the two groups in age, body mass index, mean HbA_{1c} level at onset and presence or absence of family history of type 1 or type 2 diabetes in first-degree relatives. One, but not another, allele of class II HLA haplotype was common between two patients (father and his son) with a family history of

Table 1 | Clinical characteristics of patients with fulminant type 1 diabetes

	Total	With GADab	Without GADab	Control
n	207	25 (12.1)	182 (87.9)	325
Sex (male/female)	118/89 (57.0)	20/5 (80.0)	98/84 (53.8)	202/123 (62.2)
Pregnancy (PF*/NPF†)	15/51 (22.7)	0/5 (0.0)	15/49 (23.4)	ND
Age at disease onset (years)	41 (0–87)	43 (0–75)	41 (1–87)	47 (25–78)
Body mass index (kg/m^2)	21.1 ± 3.2‡	20.9 ± 3.4§	21.2 ± 3.2¶	ND
Family history of type 1 diabetes	5/157 (3.1)	0/20 (0.0)	5/137 (3.5)	0/0 (0.0)
Family history of type 2 diabetes	11/151 (6.8)	2/18 (10.0)	9/133 (6.3)	0/0 (0.0)
Family history of unclassified diabetes	6/156 (3.7)	1/19 (5.0)	5/137 (3.5)	0/0 (0.0)
HbA_{1c} at disease onset (%)	6.6 ± 0.8	6.7 ± 0.7	6.6 ± 0.8	ND

GADab, antibodies to glutamic acid decarboxylase; ND, not determined.
Data are *n*, median (range), mean ± SD, (±), or *n* (%).
*Pregnancy-associated fulminant type 1 diabetes; †Female patients of child-bearing age (13–49 years) with fulminant type 1 diabetes not associated with pregnancy; ‡except seven children; §Except two children; ¶Except five children.

fulminant type 1 diabetes. GADab was measured by radioimmunoassay[1], except for one patient in whom GADab was measured by radioligand binding assay[25]. There were no differences in sensitivity and specificity between the two assays. GADab was determined within a week after the onset of diabetes, except for two patients in each hospital. GADab was negative in one patient measured 6 months after the onset and positive in another patient measured 16 years after the onset. The median level of GADab was 3.0 U/mL (range 1.5–20.0 U/mL). In 78% of GADab-positive patients, the titer was <10 U/mL at the onset of disease and GADab became negative within 2 years during the follow up. Of 25 GADab-positive patients with fulminant type 1 diabetes, IA-2ab was negative in 16 patients and not measured in the other nine patients. In GADab-positive patients with fulminant type 1 diabetes, median duration of hyperglycemic symptoms was 4 days (range 0–11 days); median HbA_{1c} level was 6.7% (range 5.6–8.3%) despite very high plasma glucose levels (median 700, range 313–1944 mg/dL), showing the similarity in the clinical features, except the positivity of GADab, between GADab-positive and GADab-negative fulminant type 1 diabetes.

Table 2 | *DRB1* and *DQB1* alleles in patients with fulminant type 1 diabetes and control subjects

		Fulminant			Control (n = 650†)	Total vs control		GADab(+) vs control		GADab(−) vs control	
		Total (n = 414†)	GADab(+) (n = 50†)	GADab(−) (n = 364†)							
		n (%)	n (%)	n (%)	n (%)	Pc	OR	Pc	OR	Pc	OR
DRB1	**01:01*	9 (2.2)	0 (0.0)	9 (2.5)	50 (7.7)	2.8×10^{-3}	0.27	NS		0.015	0.30
	**04:01*	6 (1.4)	1 (2.0)	5 (1.4)	5 (0.8)	NS		NS		NS	
	**04:03*	6 (1.4)	1 (2.0)	5 (1.4)	21 (3.2)	NS		NS		NS	
	**04:05*	135 (32.6)	11 (22.0)	124 (34.0)	92 (14.2)	1.7×10^{-11}	2.9	NS		2.4×10^{-12}	3.1
	**04:06*	3 (0.7)	1 (2.0)	2 (0.5)	23 (3.5)	NS		NS		NS	
	**04:07*	1 (0.2)	0 (0.0)	1 (0.3)	5 (0.8)	NS		NS		NS	
	**04:10*	13 (3.1)	0 (0.0)	13 (3.6)	9 (1.4)	NS		NS		NS	
	**08:02*	14 (3.4)	2 (4.0)	12 (3.3)	30 (4.6)	NS		NS		NS	
	**08:03*	13 (3.1)	1 (2.0)	12 (3.3)	58 (8.9)	5.0×10^{-3}	0.33	NS		0.015	0.35
	**09:01*	106 (25.6)	22 (44.0)	84 (23.1)	90 (13.8)	3.1×10^{-5}	2.1	4.6×10^{-7}	4.9	4.1×10^{-3}	1.9
	**10:01*	1 (0.2)	0 (0.0)	1 (0.3)	9 (1.4)	NS		NS		NS	
	**11:01*	3 (0.7)	0 (0.0)	3 (0.8)	13 (2.0)	NS		NS		NS	
	**12:01*	7 (1.7)	1 (2.0)	6 (1.6)	27 (4.2)	NS		NS		NS	
	**12:02*	5 (1.2)	2 (4.0)	3 (0.8)	9 (1.4)	NS		NS		NS	
	**13:02*	23 (5.6)	1 (2.0)	22 (6.0)	26 (4.0)	NS		NS		NS	
	**14:01*	7 (1.7)	1 (2.0)	6 (1.6)	23 (3.5)	NS		NS		NS	
	**14:03*	1 (0.2)	0 (0.0)	1 (0.3)	6 (0.9)	NS		NS		NS	
	**14:05*	3 (0.7)	0 (0.0)	3 (0.8)	12 (1.8)	NS		NS		NS	
	**14:06*	3 (0.7)	0 (0.0)	3 (0.8)	7 (1.1)	NS		NS		NS	
	**15:01*	22 (5.3)	3 (6.0)	19 (5.2)	45 (6.9)	NS		NS		NS	
	**15:02*	16 (3.9)	0 (0.0)	16 (4.4)	73 (11.2)	5.1×10^{-4}	0.32	NS		4.9×10^{-3}	0.36
	**16:02*	8 (1.9)	2 (4.0)	6 (1.6)	6 (0.9)	NS		NS		NS	
	Others	9 (2.2)	1 (2.0)	8 (2.2)	11 (1.6)						
DQB1	**03:01*	18 (4.3)	3 (6.0)	15 (4.1)	62 (9.5)	0.019	0.43	NS		0.020	0.41
	**03:02*	21 (5.1)	2 (4.0)	19 (5.2)	67 (10.3)	0.028	0.46	NS		NS	
	**03:03*	109 (26.3)	22 (44.0)	87 (23.9)	97 (14.9)	4.9×10^{-5}	2.0	1.5×10^{-6}	4.5	4.1×10^{-3}	1.8
	**04:01*	133 (32.1)	11 (22.0)	122 (33.5)	91 (14.0)	1.7×10^{-11}	2.9	NS		2.8×10^{-12}	3.1
	**04:02*	22 (5.3)	2 (4.0)	20 (5.5)	27 (4.2)	NS		NS		NS	
	**05:01*	11 (2.7)	0 (0.0)	11 (3.0)	59 (9.1)	4.2×10^{-4}	0.27	NS		2.9×10^{-3}	0.31
	**05:02*	11 (2.7)	3 (6.0)	8 (2.2)	19 (2.9)	NS		NS		NS	
	**05:03*	8 (1.9)	1 (2.0)	7 (1.9)	23 (3.5)	NS		NS		NS	
	**06:01*	30 (7.2)	1 (2.0)	29 (8.0)	132 (20.3)	8.1×10^{-8}	0.31	0.030	0.08	2.7×10^{-6}	0.34
	**06:02*	21 (5.1)	3 (6.0)	18 (4.9)	44 (6.8)	NS		NS		NS	
	**06:04*	20 (4.8)	1 (2.0)	19 (5.2)	26 (4.0)	NS		NS		NS	
	Others	10 (2.4)	1 (2.0)	9 (2.5)	4 (0.6)						

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.
Pc-, *P*-values corrected for number of different alleles tested (×22 for *DRB1*and ×11 for *DQB1*).
†Allele number.

Frequencies of Alleles of HLA-DRB1 and DQB1

As shown in Table 2, the allele frequencies of *DRB1*04:05*, *DRB1*09:01*, *DQB1*04:01* and *DQB1*03:03* were significantly higher, and those of *DRB1*01:01*, *DRB1*08:03*, *DRB1*15:02*, *DQB1*03:01*, *DQB1*03:02*, *DQB1*05:01* and *DQB1*06:01* were significantly lower in total subjects with fulminant type 1 diabetes than in control subjects.

Similarly, the allele frequencies of *DRB1*04:05*, *DRB1*09:01*, *DQB1*04:01* and *DQB1*03:03* were significantly higher, and those of *DRB1*01:01*, *DRB1*08:03*, *DRB1*15:02*, *DQB1*03:01*, *DQB1*05:01* and *DQB1*06:01* were significantly lower in GADab-negative patients with fulminant type 1 diabetes than in control subjects.

In contrast, the allele frequencies of *DRB1*09:01* and *DQB1*03:03* were significantly higher, and that of *DQB1*06:01* was significantly lower in GADab-positive patients with fulminant type 1 diabetes than in control subjects (Table 2).

The frequencies of *DRB1*09:01* and *DQB1*03:03* were significantly higher in GADab-positive patients than in GADab-negative patients with fulminant type 1 diabetes (44.0 vs 23.1%, $Pc = 0.033$ and 44.0 vs 23.9%, $Pc = 0.027$, respectively).

Frequencies of the Genotypes of DRB1-DQB1 Haplotypes

As shown in Table 3, *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* are significantly more frequent in total subjects with fulminant type 1 diabetes than in controls. *DRB1*15:02-DQB1*06:01*, but not *DRB1*15:01-DQB1*06:02*, was significantly less frequent in these patients than in control subjects. Furthermore, *DRB1*01:01-DQB1*05:01* and *DRB1*08:03-DQB1*06:01* were significantly less frequent in these patients than in controls.

Similarly, the frequencies of *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* were significantly higher and those of *DRB1*01:01-DQB1*05:01*, *DRB1*15:02-DQB1*06:01* and *DRB1*08:03-DQB1*06:01* were significantly lower in

Table 3 | *DRB1-DQB1* haplotypes in patients with fulminant type 1 diabetes and control subjects

DRB1-DQB1	Fulminant			Control (n = 650†)	Total vs control		GADab(+) vs control		GADab(−) vs control	
	Total (n = 414†)	GADab(+) (n = 50†)	GADab(−) (n = 364†)							
	n (%)	n (%)	n (%)	n (%)	Pc	OR	Pc	OR	Pc	OR
**01:01-*05:01*	9 (2.2)	0 (0.0)	9 (2.5)	50 (7.7)	3.1×10^{-3}	0.27	NS	0.12	0.016	0.30
**04:01-*03:01*	3 (0.7)	1 (2.0)	2 (0.5)	5 (0.7)	NS		NS		NS	
**04:03-*03:02*	6 (1.4)	1 (2.0)	5 (1.4)	22 (3.2)	NS		NS		NS	
**04:05-*04:01*	135 (32.6)	11 (22.0)	124 (34.1)	92 (14.2)	2.0×10^{-11}	2.9	NS	1.7	2.7×10^{-12}	3.1
**04:06-*03:02*	3 (0.7)	1 (2.0)	2 (0.5)	23 (3.5)	NS		NS		NS	
**04:07-*03:02*	1 (0.2)	0 (0.0)	1 (0.3)	5 (0.7)	NS		NS		NS	
**04:10-*04:02*	13 (3.1)	0 (0.0)	13 (3.6)	9 (1.3)	NS		NS		NS	
**08:02-*03:02*	6 (1.4)	0 (0.0)	6 (1.6)	15 (2.2)	NS		NS		NS	
**08:02-*04:02*	8 (1.9)	2 (4.0)	6 (1.6)	16 (2.3)	NS		NS		NS	
**08:03-*06:01*	13 (3.1)	1 (2.0)	12 (3.3)	58 (8.9)	5.7×10^{-3}	0.33	NS	0.21	0.017	0.35
**09:01-*03:03*	105 (25.4)	22 (44.0)	83 (22.8)	89 (13.7)	3.8×10^{-5}	2.1	3.9×10^{-7}	5.0	5.2×10^{-3}	1.9
**10:01-*05:01*	1 (0.2)	0 (0.0)	1 (0.3)	10 (1.5)	NS		NS		NS	
**11:01-*03:01*	1 (0.2)	0 (0.0)	1 (0.3)	13 (1.9)	NS		NS		NS	
**12:01-*03:01*	4 (1.0)	1 (2.0)	3 (0.8)	21 (3.1)	NS		NS		NS	
**12:01-*03:03*	2 (0.5)	0 (0.0)	2 (0.5)	5 (0.7)	NS		NS		NS	
**12:02-*03:01*	4 (1.0)	1 (2.0)	3 (0.8)	9 (1.3)	NS		NS		NS	
**13:02-*06:04*	19 (4.6)	1 (2.0)	18 (4.9)	23 (3.5)	NS		NS		NS	
**14:01-*05:02*	2 (0.5)	0 (0.0)	2 (0.5)	13 (1.9)	NS		NS		NS	
**14:01-*05:03*	5 (1.2)	1 (2.0)	4 (1.1)	13 (1.9)	NS		NS		NS	
**14:03-*03:01*	1 (0.2)	0 (0.0)	1 (0.3)	6 (0.9)	NS		NS		NS	
**14:05-*05:03*	3 (0.7)	0 (0.0)	3 (0.8)	13 (1.9)	NS		NS		NS	
**14:06-*03:01*	3 (0.7)	0 (0.0)	3 (0.8)	7 (1.0)	NS		NS		NS	
**15:01-*06:02*	20 (4.8)	3 (6.0)	17 (4.7)	43 (6.6)	NS		NS		NS	
**15:02-*06:01*	16 (3.9)	0 (0.0)	16 (4.4)	79 (11.2)	9.5×10^{-5}	0.29	NS	0.07	1.2×10^{-3}	0.33
**16:02-*05:02*	7 (1.7)	2 (4.0)	5 (1.4)	6 (0.9)	NS		NS		NS	
Others	24 (5.8)	2 (4.0)	22 (6.0)	20 (2.9)						

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.
Pc-, *P*-values corrected for number of different haplotypes tested (×25).
†Allele number.

GADab-negative patients with fulminant type 1 diabetes than in control subjects.

In contrast, only *DRB1*09:01-DQB1*03:03* was significantly more frequent in GADab-positive patients with fulminant type 1 diabetes than in controls. The frequency of *DRB1*09:01-DQB1*03:03* was significantly higher (44.0 vs 22.8%, *Pc* = 0.031) in GADab-positive patients than in GADab-negative patients with fulminant type 1 diabetes.

Comparison between DRB1*04:05-DQB1*04:01 and DRB1*09:01-DQB1*03:03 Haplotypes

To clarify the difference in the genetic contribution of the two major HLA haplotypes, *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*, to fulminant type 1 diabetes, we analyzed the frequencies of homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* and/or *DRB1*09:01-DQB1*03:03* in patients with this form of diabetes and control subjects. As shown in Table 4, homozygotes with both *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* were significantly more frequent in total subjects of fulminant type 1 diabetes than in control subjects. Heterozygotes with *DRB1*04:05-DQB1*04:01*, but not *DRB1*09:01-DQB1*03:03*, were also significantly more frequent in these patients than in control subjects.

Similarly, both homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* were significantly more frequent in GADab-negative patients with fulminant type 1 diabetes than in control subjects. Homozygotes, but not heterozygotes, with *DRB1*09:01-DQB1*03:03* were present significantly more frequently in GADab-negative patients than in control subjects.

In contrast, both homozygotes and heterozygotes with *DRB1*09:01-DQB1*03:03* were significantly more frequent in GADab-positive patients with fulminant type 1 diabetes than in control subjects. Furthermore, neither homozygotes nor heterozygotes with *DRB1*04:05-DQB1*04:01* were associated with GADab-positive patients with fulminant type 1 diabetes.

When analyzed by using a 2 × 3 contingency table (homozygote, heterozygote and null of *DRB1*04:05-DQB1*04:01* or *DRB1*09:01-DQB1*03:03* between GADab-positive and GADab-negative patients; Table 4), there was a significant difference in the frequency of *DRB1*09:01-DQB1*03:03* ($P = 0.0093$), but not in the frequency of *DRB1*04:05-DQB1*04:01* ($P = 0.29$), between GADab-positive and GADab-negative patients.

To further investigate the disease susceptibility and protection provided by HLA haplotypes in fulminant type 1 diabetes, we examined the genotypic combinations classified as high-frequency haplotypes (*DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*) and low-frequency haplotypes (*DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01*) in patients with fulminant type 1 diabetes and in control subjects. As shown in Table 5, none of low-frequency haplotypes, such as *DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01*, conferred protection to fulminant type 1 diabetes in combination with high-frequency haplotypes, such as *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*, although the number of patients was small.

Frequencies of the Genotypes of DRB1-DQB1 Haplotypes in Pregnancy

*DRB1*04:05-DQB1*04:01* was found to be significantly more frequent in the NPF group than in control subjects, whereas *DRB1*09:01-DQB1*03:03* was not significantly more frequent in either PF or NPF group compared with the controls (Table S1).

Homozygotes with *DRB1*04:05-DQB1*04:01* were significantly more frequent in the NPF group than in control subjects (Table S2). The frequency of homozygotes with *DRB1*04:05-DQB1*04:01* tended to be lower in the PF group than in the NPF group, but there was no significant difference between the groups. In contrast, neither homozygotes nor heterozygotes with *DRB1*09:01-DQB1*03:03* were associated with either the PF or NPF groups compared with the controls.

Table 4 | Combination of HLA-*DRB1-DQB1* haplotype in patients with fulminant type 1 diabetes and control subjects

DRB1-DQB1/DRB1-DQB1	Fulminant			Control ($n = 325$)	Total vs control		GADab(+) vs control		GADab(−) vs control	
	Total ($n = 207$)	GADab(+) ($n = 25$)	GADab(−) ($n = 182$)							
	n (%)	*n* (%)	*n* (%)	*n* (%)	*Pc*	OR	*Pc*	OR	*Pc*	OR
**04:05-*04:01/*04:05-*04:01*	31 (15.0)	2 (8.0)	29 (15.9)	8 (2.5)	2.0×10^{-7}	7.0	NS	3.4	6.6×10^{-8}	7.5
**04:05-*04:01/X*	73 (35.3)	7 (28.0)	66 (36.3)	76 (23.4)	8.8×10^{-3}	1.8	NS	1.3	5.8×10^{-3}	1.9
X/X	103 (49.8)	16 (64.0)	87 (47.8)	241 (74.2)	2.9×10^{-8}	0.35	NS	0.62	7.8×10^{-9}	0.32
**09:01-*03:03 *09:01-*03:03*	22 (10.6)	5 (20.0)	17 (9.3)	4 (1.2)	8.0×10^{-6}	9.5	1.3×10^{-6}	20.1	9.4×10^{-5}	8.3
**09:01-*03:03/Y*	61 (29.5)	12 (48.0)	49 (26.9)	81 (24.9)	NS	1.3	0.035	2.8	NS	1.1
Y/Y	124 (59.9)	8 (32.0)	116 (63.7)	240 (73.8)	2.2×10^{-3}	0.53	2.8×10^{-5}	0.17	NS	0.62

NS, not significant; GADab, antibodies to glutamic acid decarboxylase.
Pc-, *P*-values corrected for number of different haplotypes tested. *X* does not contain *DRB1*04:05-DQB1*04:01*. *Y* does not contain *DRB1*09:01-DQB1*03:03*.

Table 5 | Genotypic combination of *DRB1-DQB1* haplotype in patients with fulminant type 1 diabetes and control subjects

Allele frequency High/Low *DRB1-DQB1/ DRB1-DQB1*	Fulminant			Control (n = 325)	Total vs control		GADab(+) vs control		GADab(−) vs control	
	Total (n = 207)	GADab(+) (n = 25)	GADab(−) (n = 182)							
	n (%)	n (%)	n (%)	n (%)	P	OR	P	OR	P	OR
**04:05-*04:01/*										
**01:01-*05:01*	2 (1.0)	0 (0.0)	2 (1.1)	10 (2.9)	NS	0.31	NS	0.59	NS	0.35
**08:03-*06:01*	4 (1.9)	0 (0.0)	4 (2.2)	4 (1.2)	NS	1.6	NS	1.4	NS	1.8
**15:02-*06:01*	5 (2.4)	0 (0.0)	5 (2.7)	9 (2.6)	NS	0.87	NS	0.65	NS	0.99
**09:01-*03:03/*										
**01:01-*05:01*	1 (0.5)	0 (0.0)	1 (0.5)	7 (2.0)	NS	0.22	NS	0.83	NS	0.25
**08:03-*06:01*	4 (1.9)	0 (0.0)	4 (2.2)	7 (2.0)	NS	0.90	NS	0.83	NS	1.0
**15:02-*06:01*	2 (1.0)	0 (0.0)	2 (1.1)	15 (4.6)	0.037	0.20	NS	0.39	NS	0.23

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.

DISCUSSION

The two important findings obtained from the present study were as follows: (i) the contribution of HLA genes to fulminant type 1 diabetes was clearly shown in a large-scale study; and (ii) the contribution of HLA genes to fulminant type 1 diabetes was different between GADab-positive and GADab-negative patients.

First, the present large-scale study has clarified the contribution of HLA genes to fulminant type 1 diabetes. We have reconfirmed that *DRB1*04:05-DQB1*04:01*, but not *DRB1*04:10-DQB1*04:02*, which also encodes DR4-DQ4, confers a strong predisposition to fulminant type 1 diabetes. Analysis of the combination of the HLA-*DRB1-DQB1* haplotype has shown that both homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* show a strong effect regarding predisposition to fulminant type 1 diabetes (OR 7.0 and 1.8, respectively), as shown in a previous nationwide multicenter study[12,13]. [Correction to previous sentence, added after online publication 29 July 2011: "OR 6.4 and 1.9" is changed to "OR 7.0 and 1.8".] These findings suggest that *DRB1*04:05-DQB1*04:01* plays an important role in the development of fulminant type 1 diabetes.

We have also shown that the *DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01* haplotypes are negatively associated with fulminant type 1 diabetes. It is well known that both haplotypes of *DRB1*15:02-DQB1*06:01* and *DRB1*15:01-DQB1*06:02* encode DR2-DQ1[26]. In a previous study, we analyzed the serological subtype of HLA-DR-DQ and showed that the frequency of DR2-DQ1 was significantly lower in fulminant type 1 diabetes than in the control[12]. The present study has shown that *DRB1*15:02-DQB1*06:01*, but not *DRB1*15:01-DQB1*06:02*, which encode DR2-DQ1, was negatively associated with fulminant type 1 diabetes. Regarding the combination analysis, in the Japanese population, protective haplotypes, such as *DRB1*15:01-DQB1*06:02* and *DRB1*15:02-DQB1*06:01*, provide strong protection against type 1A diabetes regardless of the presence of susceptible haplotypes, such as *DRB1*09:01-DQB1*03:03* and *DRB1*04:05-DQB1*04:01*[13,26–31]. However, no such protective effect was observed in fulminant type 1 diabetes. This might show that protective haplotypes are not superior to susceptible haplotypes in fulminant type 1 diabetes.

*DRB1*09:01-DQB1*03:03*, in addition to *DRB1*04:05-DQB1*04:01*, haplotype was positively associated with fulminant type 1 diabetes. Recently, we have reported the differences in the contribution of HLA to genetic susceptibility to three subtypes of Japanese type 1 diabetes, acute-onset, fulminant and slowly-progressive, and that *DRB1*04:05-DQB1*04:01*, but not *DRB1*09:01-DQB1*03:03*, was associated with fulminant type 1 diabetes[13]. However, *DRB1*09:01-DQB1*03:03* was also high in frequency in the present study. We have two hypotheses to explain this discrepancy. One is that the maximum number of samples in the present study enabled us to re-evaluate the association of class II HLA genotype with fulminant type 1 diabetes. Another is the high frequency of *DRB1*09:01-DQB1*03:03* haplotype in GADab-positive patients with fulminant type 1 diabetes included in the present study. *DRB1*09:01-DQB1*03:03* conferred strong susceptibility to GADab-positive fulminant type 1 diabetes (OR 5.0). In addition, it has been reported that *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, confers strong susceptibility to the disease development in pregnancy-associated fulminant type 1 diabetes in Japanese[32]. A similar trend was also observed in the present study, although the difference was not significant.

Second, the present study has clarified that the contribution of HLA genes to fulminant type 1 diabetes was different between GADab-positive and GADab-negative patients despite the similar clinical status. In the present large-scale study, the majority of fulminant type 1 diabetes, GADab-negative patients, was characterized by the predominance of *DRB1*04:05-DQB1*04:01* both in homozygous and heterozygous states. In contrast, *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, was predominant in GADab-positive patients with fulminant type 1 diabetes. In addition, the protective effect of the *DRB1*15:02-DQB1*06:01* haplotype tended to be stronger in GADab-positive (0.0%, OR 0.07) than in GADab-negative

fulminant type 1 diabetes (4.4%, OR 0.33). In contrast, it is well known that the *DRB1*09:01-DQB1*03:03* haplotype is frequent in GADab-positive or typical autoimmune diabetic patients in Japan[13,27,28,31]. Kawabata *et al.* showed that the *DRB1*09:01-DQB1*03:03* haplotype confers much stronger susceptibility to Japanese typical autoimmune type 1 diabetes when present in a homozygous state and that the *DRB1*09:01-DQB1*03:03* haplotype predisposes in a recessive fashion. *DRB1*15:02-DQB1*06:01* also shows strong protection to classical type 1A diabetes[30]. High frequency of *DRB1*09:01-DQB1*03:03* homozygous state was also observed in GADab-positive fulminant type 1 diabetes in the present study (OR 20.1). Taken together, these findings suggest the similarity in underlying genetic backgrounds between classical autoimmune type 1 diabetes and GADab-positive fulminant type 1 diabetes, but not GADab-negative fulminant type 1 diabetes.

In conclusion, the present large-scale study showed the characteristic class II HLA genotypes in fulminant type 1 diabetes. The present study also implied that genetic contribution to disease susceptibility is distinct between GADab-positive and GADab-negative fulminant type 1 diabetes. Consequently, this disorder might be heterogeneous, as reflected by class II HLA and GADab, and further divided into at least two subtypes.

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APPENDIX

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SUPPORTING INFORMATION

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

Table S1 | *DRB1-DQB1* haplotypes in female patients with PF and NPF and in control subjects
Table S2 | Combination of HLA-*DRB1-DQB1* haplotype in female patients with PF and NPF and in control subjects

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Low CTLA-4 expression in CD4$^+$ helper T-cells in patients with fulminant type 1 diabetes

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ABSTRACT

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes characterized by a remarkably abrupt onset of insulin-deficient hyperglycemia. An accelerated immune reaction has been suggested as the cause of markedly rapid beta cell loss in this disease, but the precise mechanism has not been clarified. We analyzed the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) in CD4$^+$ helper T-cells in 16 patients with fulminant type 1 diabetes, 14 patients with type 1A diabetes, 10 patients with type 2 diabetes and 20 normal control subjects. There was a significant reduction in CTLA-4 expression in CD4$^+$ helper T-cells from patients with fulminant type 1 diabetes ($P<0.05$) compared with the other three groups. Low CTLA-4 expression was also observed in both CD4$^+$CD25^{high} T-cells and CD4$^+$CD25$^-$ T-cells. There was a significant negative correlation between the proliferation of CD4$^+$CD25$^-$ T-cells and the levels of CTLA-4. Intracellular expression of CTLA-4 in CD4$^+$ helper T-cells was not correlated with two *CTLA-4* polymorphisms. In conclusion, the expression of CTLA-4 in CD4$^+$ helper T-cells was low in patients with fulminant type 1 diabetes.

1. Introduction

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes characterized by almost complete insulin deficiency resulting from the destruction of pancreatic beta cells [1,2]. The clinical characteristics of this disease are as follows: (1) remarkably abrupt onset; (2) very short duration of diabetic symptoms; (3) acidosis at the time of diagnosis; (4) negative findings in general for islet-related autoantibodies, such as anti-GAD antibodies (GAD Ab), insulin autoantibodies and anti-insulinoma-associated antigen 2 antibodies (IA-2 Ab); (5) virtually no C-peptide secretion; and (6) elevated serum pancreatic enzyme levels [1–4]. Of these, the abrupt onset and very short duration suggest that rapid loss of beta cells occurs in fulminant type 1 diabetes. In addition, massive cellular infiltration of T-cells and macrophages has been detected in the pancreas just after disease onset [5,6]. Increased T-cell responses against pancreatic beta cell antigens as detected by enzyme-linked immunospot (ELISPOT) assay have been proposed [7]. Based on these findings, we hypothesize that pancreatic beta cells could be destroyed by an accelerated immune response followed by T-cell activation, leading to the development of fulminant type 1 diabetes.

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory immunoregulatory molecule that has an important role in adjusting the threshold for T-cell activation and preventing autoimmunity. Usually, CTLA-4 is only present on the surface of activated T-cells at low levels; the majority of CTLA-4 proteins is instead localized in intracellular compartments and is transported to the cell surface only in response to T-cell receptor ligation [8,9]. CTLA-4 function is mediated via both cell-extrinsic and cell-intrinsic mechanisms [10,11]. One cell-extrinsic mechanism is that regulatory T-cells (Tregs) suppress the proliferation of effector T-cells via the CTLA-4/B7 receptor/ligand system on antigen presenting cells (APCs). Phenotypic characterization of Tregs indicates that they are CD4$^+$ T cells that co-express constitutively high levels of interleukin (IL)-2R alpha (CD25) and the transcription factor forkhead box P3 (FOXP3) in addition to CTLA-4 [12–15]. In a murine study, Treg-specific CTLA-4 deficiency impaired the suppressive function of Tregs resulting in fatal systemic T-cell lymphoproliferation; in particular, Tregs mediated the down-regulation of CD80 and CD86 expression on APCs [16]. A cell-intrinsic mechanism that regulates CTLA-4 function is mediated by binding to the intracellular phosphatases (PTPases) SRC homology 2-domain-containing PTPase 2 and protein phosphatase 2A, and these PTPases could act to inhibit the function of T cell receptor (TCR) signaling tar-

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gets through dephosphorylation [10]. Indeed, CTLA-4 regulates the activation of effector T-cells independently of Tregs, as CTLA-4 expression in effector T cells in mice with a Treg-specific CTLA-4 deficiency can substantially delay the death of *CTLA-4*$^{-/-}$ mice [16].

These findings prompted us to assess the expression of CTLA-4 in CD4$^+$ helper T-cells (CD4$^+$CD25^{high} T-cells and CD4$^+$CD25$^-$ T-cells), the frequency of CD3$^+$FOXP3$^+$ T-cells and the function of CD4$^+$CD25^{high} T-cells for their role in accelerated beta cell death in patients with fulminant type 1 diabetes.

2. Materials and methods

2.1. Research design

We studied 16 patients with fulminant type 1 diabetes (Table 1) diagnosed according to the following inclusion criteria proposed by the Committee of the Japan Diabetes Society: (1) occurrence of diabetic ketosis or ketoacidosis soon (around 7 days) after the onset of hyperglycemic symptoms (elevation of urinary and/or serum ketone bodies at first visit); (2) plasma glucose level ≥16.0 mmol/l (≥288 mg/dl) and glycated hemoglobin (HbA1c) level <8.9% at first visit; (3) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide level <0.3 ng/ml and <0.5 ng/ml (<0.17 nmol/l) after intravenous glucagon load (or after a meal) at onset [3]. We also performed a longitudinal analysis in two patients with recent-onset and two patients with long-standing fulminant type 1 diabetes. The value for HbA1c (%) is estimated as an NGSP equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS) (%) + 0.4%, based on the relational expression of HbA1c (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [17].

As a control, we also studied 14 patients with long-standing type 1A diabetes, 10 patients with type 2 diabetes and 20 age-matched healthy control subjects (Table 1). The inclusion criteria for type 1A diabetes were as follows; (1) the presence of ketoacidosis at the onset of diabetes; (2) duration of hyperglycemic symptoms before insulin therapy of <3 months; (3) insulin replacement therapy required throughout the study period; and (4) the presence of GADAb or IA-2Ab.

This study was approved by the ethics committee of Osaka Medical College. Written informed consent was obtained from all patients.

2.2. Flow cytometric analyses

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway), and immediately subjected to cellular staining. A total of 1×10^6 PBMCs was aliquoted (per tube) along with 20 μl of each appropriate test antibody or the respective isotype control. The cells were stained with the following antibodies: fluorescein isothiocyanate (FITC) anti-CD3 (clone HIT3a), phycoerythrin (PE) anti-CD25 (clone M-A251), PE anti-CD127 (clone hIL-7R-M21), allophycocyanin (APC)-CD152 (clone BNI3), APC-Cy™7-anti-CD25 (clone M-A251), peridinin chlorophyll protein (Per CP) anti-CD4 (clone SK3) (BD Bioscience, San Jose CA) and APC-FOXP3 (clone PCH101) (eBioscience, San Diego, CA). The following isotype control antibodies were used: FITC mouse IgG1 (40×), PerCP mouse IgG1 (MOPC-21), PE mouse IgG1 (MOPC-21), and APC-Cy™7 mouse IgG1 (MOPC-21) (BD Bioscience), and APC rat IgG2a (eBR2a) (eBioscience, San Diego, CA). After surface staining for 30 min (4 °C) in the dark, the cells were washed twice with cold PBS. Surface-stained cells were then permeabilized for intracellular CTLA-4 or FOXP3 staining using the Cytofix/Cytoperm™ Fixation Permeabilization kit (BD Bioscience) or anti-human FOXP3 staining kit (eBioscience) according to the manufacturer's recommendations. Stained cells were then analyzed by flow cytometry using a BD FACSAria™ Cell Sorter (BD Bioscience). BD FACSDiva Software was used for analysis of the cytometric data. At least 100,000 events were acquired from each sample.

2.3. Isolation of RNA and quantitative real-time polymerase chain reaction

CD4$^+$ T-cells were purified from PBMC by magnetic bead isolation (BD Bioscience). Total RNA was extracted from CD4$^+$ T-cells by using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic GMBH, Mannheim, Germany). The mRNA levels were quantified using the TaqMan real-time RT-PCR method (LyghtCycler, Roche). The sequences of the primers and Roche universal probe number (#) were as follows: CTLA-4 (GenBank Accession No. L15006. 1: left primer tcacagctttctttgagca, right primer aggctgaaattgctttcaca, and #21 probe; GAPDH (NM002046): left primer agccacatcgctcagacac, right primer gcccaatacgaccaaatcc, and #60 probe.

2.4. Genotyping of CTLA-4 polymorphism

Two single nucleotide polymorphisms (SNPs) in the *CTLA-4* gene, +49G>A (rs231775) and CT60 (rs3087243), were genotyped in patients with fulminant type 1 diabetes, type 1A diabetes and normal control subjects as reported previously [18,19].

2.5. Cell isolation

PBMCs were stained with Per CP anti-CD4 (SK3) and PE anti-CD25 (M-A251). CD4$^+$CD25^{high} (CD25$^+$ top1% of CD3$^+$ T-cells) and CD4$^+$CD25$^-$ fractions were sorted with a BD FACSAria™ Cell Sorter. The sorted CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T-cell samples had purities greater than 95%. T-cell-depleted accessory cells were isolated by negative selection from PBMCs using anti-CD3 microbeads (BD Bioscience) according to the manufacturer's recommendations; these cells were by irradiated at 3000 rad. T-cell-depleted accessory cells were more than 98% pure.

2.6. Cell culture

Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with penicillin (50 μg/ml)/streptomycin (50 μg/ml) (GIBCO) and 5% human type AB serum (Gemini Bio-Products) in U-bottom 96-well plates (Becton Dickinson France S.A.).

2.7. Proliferation assay

Proliferation assays were performed by culturing sorted CD4$^+$CD25$^-$ T-cells (1×10^4/well) with CD4$^+$CD25^{high} T-cells at various ratios (0:1, 1:0, 1:1/2, and 1:1/4) in the presence of 5×10^4 irradiated accessory cells. These co-cultures were stimulated using a combination of 5 μg soluble anti-CD3 (clone HIT3a) and 5 μg soluble anti-CD28 (clone CD28.2; BD Bioscience). All culture conditions were tested in triplicate. On day 5 of the culture, 1 μ Ci [^{3}H]thymidine (MP Biomedicals, Inc.) was added for the final 16 h of culture to assess proliferation. The percentage suppression was calculated as 100 − (mean cpm of co-cultures − CD4$^+$CD25^{high} T-cells alone/mean cpm of CD4$^+$CD25$^-$ T-cells alone) × 100 at a ratio of 1:1 (CD4$^+$CD25$^-$ T-cells: CD4$^+$CD25^{high} T-cells).

Table 1
Patients demographics.

	n	Gender (m/f)	Age (years)	GAD/IA-2 antibody positive (%)	*DRB1*0405*	*DRB1*0901*	Duration (years)
Fulminant type 1 diabetes	16	7/9	45 (22–67)	0/0	6/10	2/10	3.7 (0.04–10.3)
Type 1A diabetes	14	4/10	49 (25–66)	100/60($n=5$)	8/12	3/12	7.3 (2.0–23.3)
Type 2 diabetes	10	7/3	60 (32–74)	0/ND	2/7	1/7	8.0 (0.3–24.0)
Control subjects	20	14/6	43 (27–61)	ND/ND	2/13	1/13	

Data of *DRB1*0405* and *DRB1*0901* indicate number of patients with at least one allele/total number of patients; ND, not determined.

2.8. Cytokine determination

We measured serum cytokine concentrations in 8 patients with fulminant type 1 diabetes at the time of the quantitative analysis. Cytokine levels of IL-2, IL-4, IL-6, IL-10, IL-1beta, IL-17, gamma-interferon (IFN-γ), tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) were measured by enzyme immunoassay or chemiluminescent enzyme immunoassay.

2.9. Statistical analysis

Differences between the groups were analyzed for statistical significance by the two-tailed unpaired Student's *t* test. We performed a power analysis to determine minimum number of samples required for each comparison. Power analysis was as follows: power.*t*.test (delta = 4, SD = 3, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 9.889068, power.*t*.test (delta = 3, SD = 2, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 8.06031, power.*t*.test (delta = 15, SD = 8, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 5.609417 and power.*t*.test (delta = 11, SD = 5, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 4.44319. A Spearman correlation was used to calculate the *r* value. $P < 0.05$ was considered significant.

3. Results

3.1. Intracellular CTLA-4 and CTLA-4 mRNA expression in CD4+ helper T-cells

To measure the levels of intracellular CTLA-4 expression in $CD4^+$ helper T-cells, we counted $CD4^+$ T-cells that co-expressed intracellular CTLA-4 (representative plots in Fig. 1A–C). There was a significant reduction in CTLA-4 expression in $CD4^+$ helper T-cells from patients with fulminant type 1 diabetes [median 6.8%, range 0.7–11.3%] compared with normal control subjects [median 13.5%, range 6.8–23.3%; $P = 0.0002$], patients with type 2 diabetes [median 9.0%, range 7.3–17.4%; $P = 0.0092$] and patients with type 1A diabetes [median 10.6%, range 4.6–18.8%; $P = 0.0097$] (Fig. 2A). The levels of intracellular CTLA-4 expression in $CD4^+$ helper T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together. The expression of CTLA-4 mRNA in $CD4^+$ helper T-cells was not significantly different among the patients with fulminant type 1 diabetes [median 0.853, range 0.389–24.2: $n = 7$], the group with type 1A diabetes [median 1.28, range 0.283–21.0: $n = 7$], the group with type 2 diabetes [median 0.489, range 0.133–5.58: $n = 4$] and the normal control subjects [median 1.25, range 0.671–10.9: $n = 10$] (shown in arbitrary units).

3.2. Intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells

To determine the levels of intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells, we counted $CD4^+CD25^-$ T-cells that co-expressed intracellular CTLA-4. There was a significant reduction in CTLA-4 expression in $CD4^+CD25^-$ T-cells from patients with fulminant type 1 diabetes [median 4.0%, range 1.5–7.6%] compared with normal control subjects [median 9.7%, range 5.1–18.5%; $P = 0.0003$], patients with type 2 diabetes [median 6.0%, range 4.5–13.7%; $P = 0.0126$] and patients with type 1A diabetes [median 7.1%, range 5.0–12.5%; $P = 0.0019$] (Fig. 2B). The levels of intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together.

3.3. Intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells

To determine the levels of intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells, we counted the $CD4^+CD25^{high}$ T-cells that co-expressed intracellular CTLA-4. There was a significant reduction in CTLA-4 expression in $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 1% of $CD3^+$ T-cells) from patients with fulminant type 1 diabetes [median 62.4%, range 45.2–76.6%] compared with normal control subjects [median 86.1%, range 69.3–93.1%; $P < 0.0001$], patients with type 2 diabetes [median 81.4%, range 67.5–89.5%; $P < 0.0001$] and patients with type 1A diabetes [median 75.9%, range 57.6–94.4%; $P = 0.0020$] (Fig. 2C). Reduced intracellular CTLA-4 expression was also confirmed when we counted both $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 2% of $CD3^+$ T-cells) and $CD4^+CD25^+CD127^{low}$ T-cells co-expressing intracellular CTLA-4 (Figs. 1B and 2D). The levels of intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together.

3.4. Frequency of $CD3^+FOXP3^+$ T-cells and cytokine levels

To determine the frequency of Tregs, we counted the $CD3^+$ T-cells that co-expressed intracellular FOXP3 (representative plots in Fig. S1). The frequency of $CD3^+FOXP3^+$ T-cells did not differ among patients with fulminant type 1 diabetes [median 3.51%, range 1.00–6.17%], patients with type 1A diabetes [median 3.51%, range 1.87–7.40%], and normal controls [median 3.58%, range 1.16–5.11%] (Fig. 3). The frequency of $CD3^+FOXP3^+$ T-cells in patients with type 1A and fulminant type 1 diabetes had no correlation to their age, gender, disease duration, HbA1c level, C-peptide level or insulin dose. No significant alterations were observed in patients with recent-onset or established fulminant type 1 diabetes over a period of 6 months (Fig. S2). Cytokine levels in the fulminant type 1 diabetic patients had no correlation to the frequency of $CD3^+FOXP3^+$ T-cells (Table S1).

3.5. Function of $CD4^+CD25^{high}$ T-cells assessed by their ability to suppress the proliferation of $CD4^+CD25^-$ T-cells

When we co-cultured $CD4^+CD25^-$ T-cells with $CD4^+CD25^{high}$ T-cells at various ratios (0:1, 1:0, 1:1/2, and 1:1/4), cellular proliferation was similarly suppressed in a dose-dependent manner by $CD4^+CD25^{high}$ T-cells from patients with fulminant type 1 diabetes, patients with type 1A diabetes and normal controls (Fig. 4A). No significant differences in proliferation among the three groups were observed when either $CD4^+CD25^-$ T-cells were cultured alone

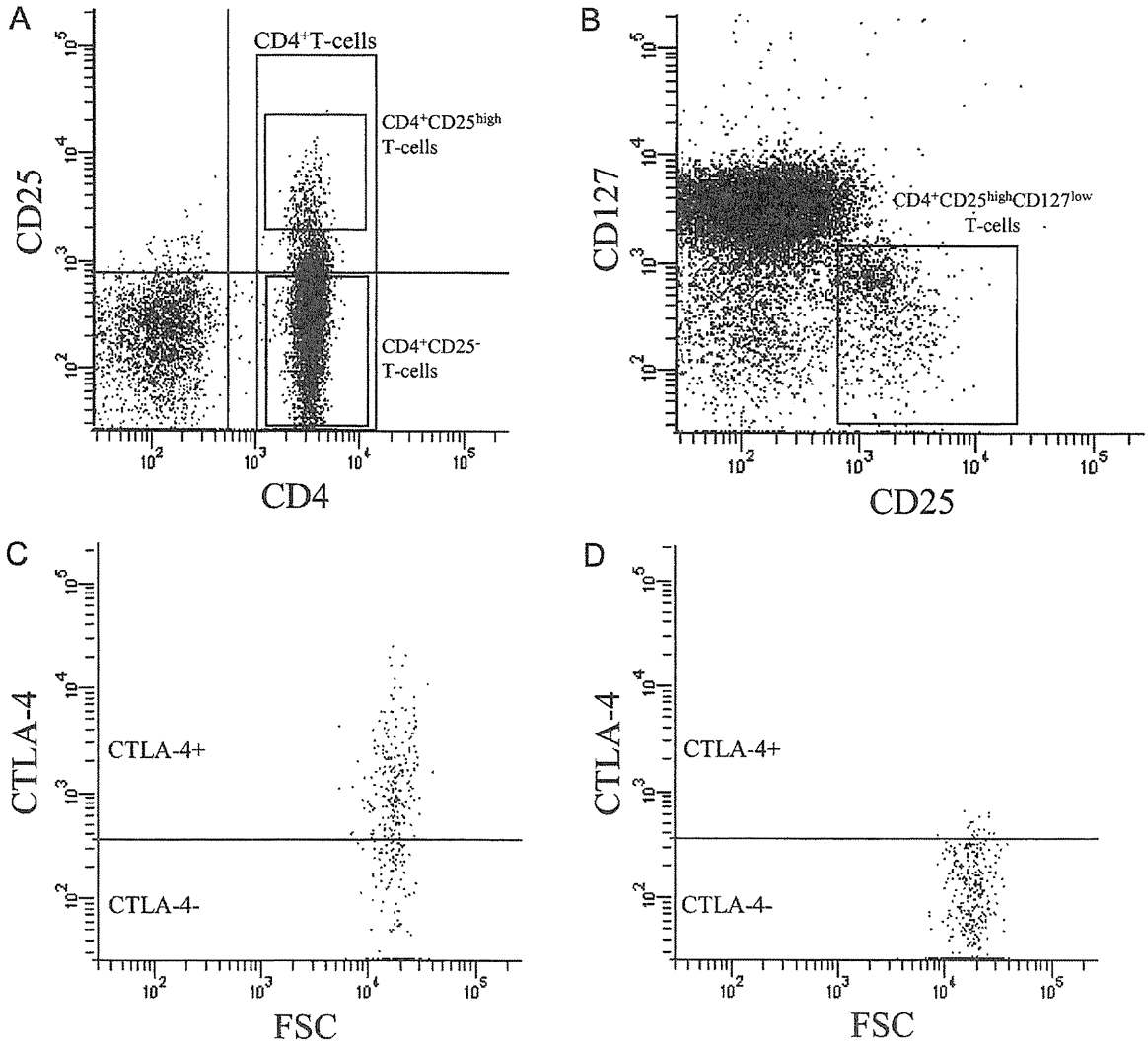

Fig. 1. Flow cytometric analysis of intracellular CTLA-4. Flow cytometric analysis of intracellular CTLA-4 from fresh peripheral blood. Representative plots showed one healthy control sample gated on lymphocytes and $CD3^+$ T-cells showing CD4 and CD25 staining (A) and CD25 and CD127 staining (B), and intracellular staining for CTLA-4 (C), as well as the isotype control (D) by gating on $CD4^+$ T-cells or $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 1% of $CD3^+$ T-cells) or $CD4^+CD25^-$ T-cells.

(0:1 ratio) or $CD4^+CD25^{high}$ T-cells were cultured alone (1:0 ratio) (Fig. 4A). The suppressive function of the $CD4^+CD25^{high}$ T-cells did not differ among the group with fulminant type 1 diabetes [median 90%, range 70–98%], the group with type 1A diabetes [median 78%, range 64–95%] and the normal control subjects [median 84.5%, range 46–95%] (Fig. 4B).

3.6. Correlation between the proliferation and the levels of CTLA-4 in $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T-cells

When we investigated the correlation between proliferation and the levels of CTLA-4 in $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T-cells in 18 subjects (7 fulminant type 1 diabetes, 5 type 1A diabetes and 6 control subjects), there was a significant negative correlation in $CD4^+CD25^-$ T-cells [$r=-0.4473$, $P<0.05$] (Fig. 5A), but not in $CD4^+CD25^{high}$ T-cells (Fig. 5B).

3.7. Correlation between CTLA-4 polymorphisms and the levels of CTLA-4 in $CD4^+$ helper T-cells

The frequency of the CTLA-4 polymorphisms (exon1 *+49* and *CT60*) is shown in Table 2. There was no correlation between those polymorphisms and the levels of CTLA-4 expression in $CD4^+$ helper T-cells either in patients with fulminant type 1 diabetes or in all subjects considered together in this study.

4. Discussion

We have clearly shown that intracellular CTLA-4 expression was reduced in $CD4^+$ helper T-cells from patients with fulminant

Table 2
CTLA-4 polymorphisms in fulminant type 1 diabetes, type 1A diabetes, type 2 diabetes and healthy control subjects.

	Fulminant type 1 diabetes	Type 1A diabetes	Type 2 diabetes	Control subjects
+49 G>A				
n	10	11	7	13
AA	0(0)	0(0)	1(14.2)	3(23.1)
AG	3(30.0)	6(54.5)	3(42.9)	3(23.1)
GG	7(70.0)	5(45.5)	3(42.9)	7(53.8)
CT60 G>A				
n	10	11	7	13
AA	0(0)	0(0)	0(0)	2(15.4)
AG	1(10.0)	2(18.2)	1(14.3)	3(61.5)
GG	9(90.0)	9(81.8)	6(85.7)	8(61.5)

Data are *n* (%).

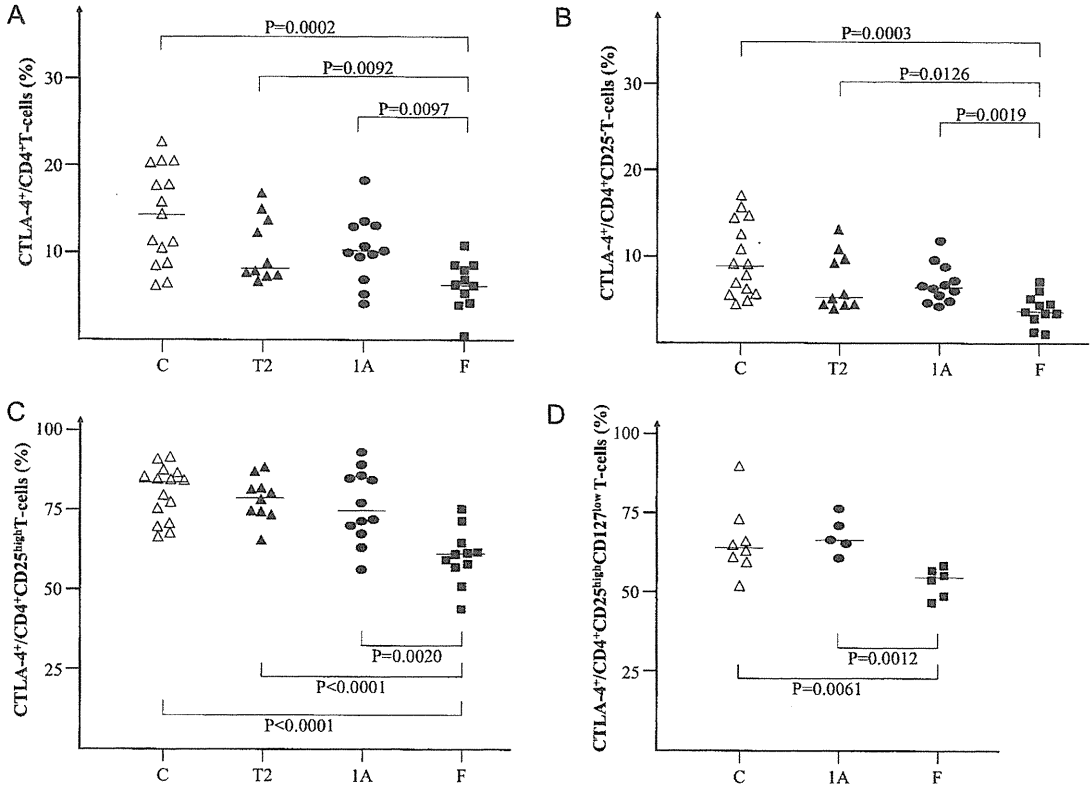

Fig. 2. Expression of intracellular CTLA-4. Reduced expression of intracellular CTLA-4 in $CD4^+$ helper T-cells (A), $CD4^+$ $CD25^-$ T-cells (B), $CD4^+CD25^{high}$ T-cells (C) and $CD4^+CD25^{high}CD127^{low}$ T-cells (D) from patients with fulminant type 1 diabetes. Data plotted represent the intracellular CTLA-4 level in each T-cell subset from normal healthy control subjects (C, open triangles), from patients with type 2 diabetes (T2, closed triangles), from patients with type 1 diabetes (1A, closed circles) and from patients with fulminant type 1 diabetes (F, closed squares). Bars represent the median.

type 1 diabetes when compared with normal control subjects, and patients with type 1A and type 2 diabetes. Reduced CTLA-4 expression was also confirmed in both $CD4^+CD25^{high}$ T-cells and $CD4^+CD25^-$ T-cells. These facts indicated that CTLA-4 would be a good diagnostic marker for fulminant type 1 diabetes. Furthermore, the reduced expression of intracellular CTLA-4 could explain the reason of the explosive immune reactivity and subsequent destruction of pancreatic beta cells observed during the clinical course of fulminant type 1 diabetes. The lack of CTLA-4 might lead to dysregulated activation of effector T-cells independently of Tregs. As far as we know, this is the first report examining intracellular CTLA-4 expression in either type 1 or type 2 diabetes that demonstrated reduced expression of the molecule in fulminant type 1 diabetes.

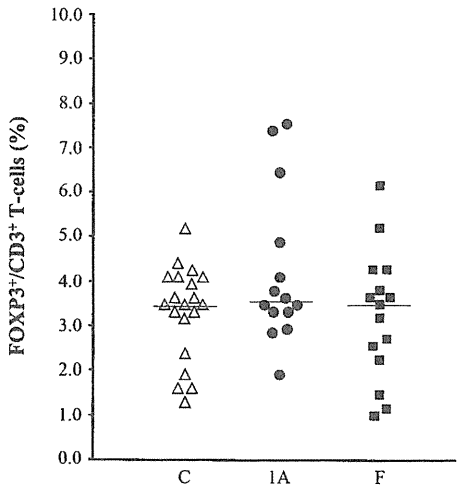

Fig. 3. Frequency of $FOXP3^+$ T-cells among $CD3^+$ T-cells. Frequency of $FOXP3^+$ T-cells (%) among $CD3^+$ T-cells from normal healthy control subjects (C, open triangles), from patients with type 1 diabetes (1A, closed circles) and from patients with fulminant type 1 diabetes (F, closed squares). Bars represent the median.

We have shown a significant reduction in CTLA-4 expression in $CD4^+CD25^-$ T-cells from patients with fulminant type 1 diabetes. There was a significant negative correlation between the proliferation of $CD4^+CD25^-$ T-cells and their levels of CTLA-4. The importance of CTLA-4 as an inhibitor of T-cell activation was clearly demonstrated using CTLA-4 knockout mice, which mice develop massive and rapidly fatal T-cell lymphoproliferation [20]. In other CTLA-4 deficient mice, irregular immune reactions were observed and beta cells were rapidly destroyed after the adoptive transfer of T cells that recognized beta-cell antigens [21]. These findings suggest that reduced expression of intracellular CTLA-4 in $CD4^+CD25^-$ T-cells might lead to an increase in the proliferation of $CD4^+CD25^-$ T-cells and result in the markedly rapid destruction of pancreatic beta cells, as seen during the clinical course of fulminant type 1 diabetes.

We have also shown a significant reduction in CTLA-4 expression in $CD4^+CD25^{high}$ T-cells from patients with fulminant type 1 diabetes. No significant correlation was observed between proliferation and the levels of CTLA-4 in $CD4^+CD25^{high}$ T-cells. This may be because $CD4^+CD25^{high}$ T-cells are themselves anergic, that is, they are unresponsive to TCR stimulation. In addition, there was no significant difference in either the frequency of $CD3^+FOXP3^+$ T-cells or the suppressive function of $CD4^+CD25^{high}$ T-cells among the three groups. An increased rate of CTLA-4 internalization may contribute to abnormal Treg function as shown in rheumatoid arthritis [22]. Further investigation is necessary to characterize the pathway leading from the reduced expression of CTLA-4 in $CD4^+CD25^{high}$ T-

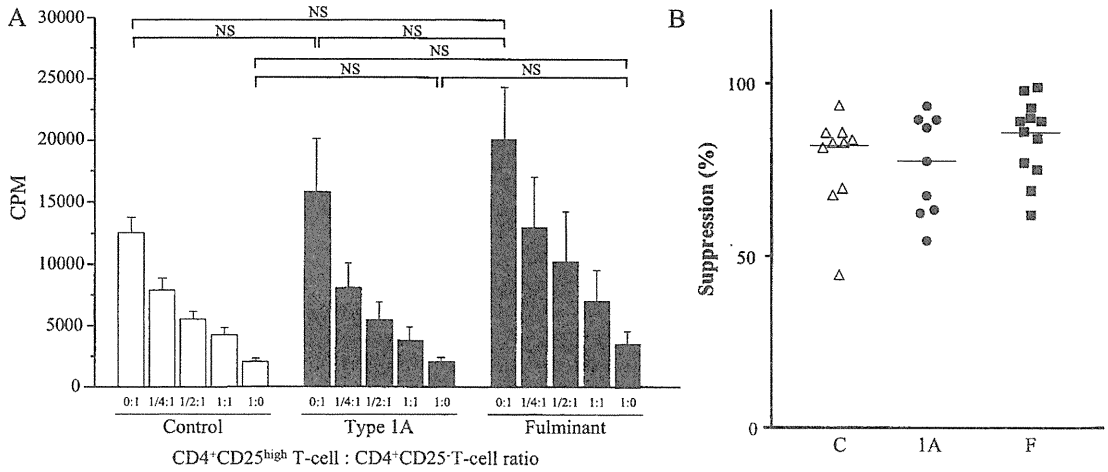

Fig. 4. Function of $CD4^+CD25^-$/$CD4^+CD25^{high}$ T-cells. (A) Proliferation in separated cell populations and coculture. Representative proliferation ([^{3}H]thymidine incorporation) of 1×10^4 $CD4^+CD25^-$ T-cells from normal healthy control subjects (open bars), from patients with type 1A diabetes (gray bars) and from patients with fulminant type 1 diabetes (closed bars) in the presence of different ratios of $CD4^+CD25^{high}$ T-cells. Bars represent the mean ±SE. (B) Function of $CD4^+CD25^{high}$ T-cells assessed by their ability to suppress the proliferation of $CD4^+CD2^-$ T-cells. Suppressive function of $CD4^+CD25^{high}$ T-cells co-cultured with $CD4^+CD25^-$ T-cells at a 1:1 ratio. Data plotted represent the percentage suppression of proliferation by $CD4^+CD25^{high}$ T-cells from normal healthy control subjects (C, open triangles), from patients with type 1 diabetes (1A, closed circles) and from patients with fulminant type 1 diabetes (F, closed squares). Bars represent the median.

cells to the accelerated immune reactivity of $CD4^+CD25^-$ T-cells in fulminant type 1 diabetes.

The mechanism behind the reduced expression of intracellular CTLA-4 was not clarified in this study. We have previously shown that the *CT60AA* subtype of *CTLA-4* was more prevalent and a lower level of soluble CTLA-4 was also found in patients with fulminant type 1 diabetes [19]. However, there was no correlation between the reduced CTLA-4 expression and either of the polymorphisms in the *CTLA-4* gene tested in this study. A low level of soluble CTLA-4 concentration in sera in our previous study [19] corresponded to reduced expression of intracellular CTLA-4 in fulminant type 1 diabetes in our present study. However, no such reduction in CTLA-4 mRNA expression was detected. Based on these findings, the reduced expression of intracellular CTLA-4 might be due to mechanisms that do not affect transcription.

In conclusion, the expression of intracellular CTLA-4 was clearly reduced in $CD4^+$ helper T-cells from patients with fulminant type 1 diabetes even after the onset of the disease. Reduced expression of CTLA-4 in $CD4^+$ helper T-cells might promote an uncontrollable immune reaction that then leads to accelerated beta cell loss and the development of fulminant type 1 diabetes.

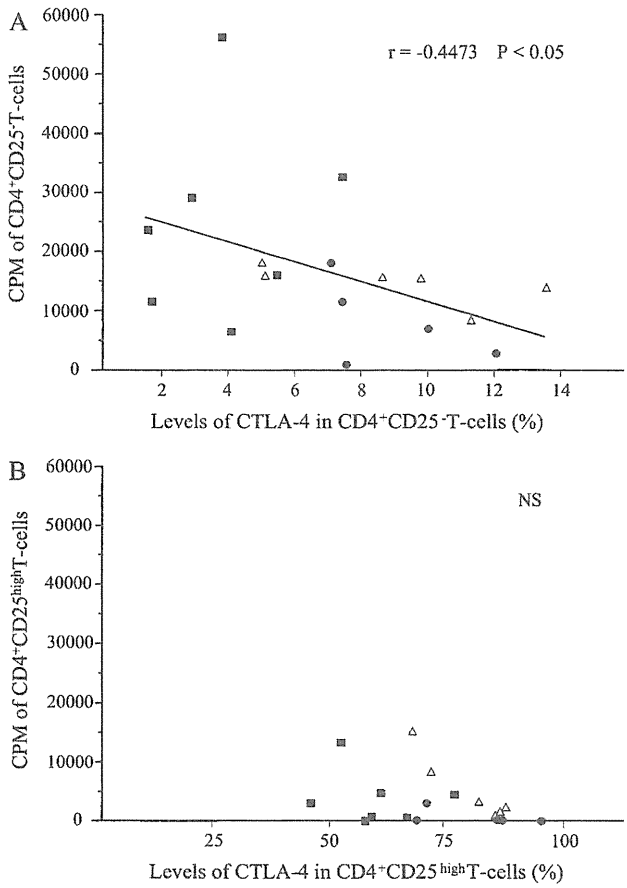

Fig. 5. Correlation between the proliferation and the levels of CTLA-4. Data plotted the correlation between the proliferation and the levels of CTLA-4 in $CD4^+CD25^-$ T-cells (A) and $CD4^+CD25^{high}$ T-cells (B) from normal healthy control subjects (open triangles), from patients with type 1 diabetes (closed circles) and from patients with fulminant type 1 diabetes (closed squares).

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2011.05.003.

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ORIGINAL ARTICLE

Computed tomography analysis of the association between the *SH2B1* rs7498665 single-nucleotide polymorphism and visceral fat area

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Visceral fat accumulation has an important role in increasing morbidity and mortality rate by increasing the risk of developing several metabolic disorders, such as type 2 diabetes, dyslipidemia and hypertension. New genetic loci that contribute to the development of obesity have been identified by genome-wide association studies in Caucasian populations. We genotyped 1279 Japanese subjects (556 men and 723 women), who underwent computed tomography (CT) for measuring visceral fat area (VFA) and subcutaneous fat area (SFA), for the following single-nucleotide polymorphisms (SNPs): *NEGR1* rs2815752, *SEC16B* rs10913469, *TMEM18* rs6548238, *ETV5* rs7647305, *GNPDA2* rs10938397, *BDNF* rs6265 and rs925946, *MTCH2* rs10838738, *SH2B1* rs7498665, *MAF* rs1424233, and *KCTD15* rs29941 and rs11084753. In the additive model, none of the SNPs were significantly associated with body mass index (BMI). The *SH2B1* rs7498665 risk allele was found to be significantly associated with VFA (P=0.00047) but not with BMI or SFA. When the analysis was performed in men and women separately, no significant associations with VFA were observed (P=0.0099 in men and P=0.022 in women). None of the other SNPs were significantly associated with SFA. Our results suggest that there is a VFA-specific genetic factor and that a polymorphism in the *SH2B1* gene influences the risk of visceral fat accumulation.
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Keywords: computed tomography; Japanese subjects; obesity; *SH2B1*; visceral fat area

INTRODUCTION

Obesity, especially visceral fat obesity, is a risk factor for several metabolic disorders, including type 2 diabetes, dyslipidemia and hypertension.[1] Several studies have indicated that adipose tissue, especially that in the visceral region, secretes various adipocytokines and that an increase in adipose tissue mass leads to alteration in the plasma levels of adipocytokines, resulting in the development of dyslipidemia, hypertension, and insulin resistance.[2,3] Intra-abdominal fat accumulation (central adiposity) is determined in terms of waist circumference; waist-hip ratio; or visceral fat area (VFA), which is measured using computed tomography (CT).[1,4,5] Recently, two genome-wide association studies were conducted to identify the loci

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linked with waist circumference and waist-hip ratio.[6,7] In a previous study, we have reported that the rs1558902 and rs1421085 genotypes of the fat mass and obesity-associated gene (*FTO*) were significantly associated with VFA, as well as with subcutaneous fat area (SFA) and body mass index (BMI).[8]

We performed a large-scale, case–control association study and found that secretogranin III (*SCG3*)[9] and myotubularin-related protein 9 (*MTMR9*)[10] conferred susceptibility to an obese phenotype in the Japanese population. Recent progress in genome-wide association studies has increased the number of known genetic susceptibility loci for obesity.[11–13] Some of the obesity-associated loci identified by the genome-wide association studies were found to be replicated in the Japanese population.[14,15] Some of the obesity-related loci were found to overlap with the waist circumference waist-hip ratio-related loci, for example, the loci within the *FTO* gene and near the melanocortin 4 receptor (*MC4R*) gene.

In this study, we investigated whether the recently reported obesity-related loci were associated with VFA, which is an important factor responsible for increased morbidity and mortality rates.

MATERIALS AND METHODS

Study subjects

In this study, we enrolled 1279 Japanese subjects from outpatient clinics; these patients had agreed to undergo CT testing (in the supine position) to determine the VFA and SFA values at the umbilical level (L4–L5). Both VFA and SFA values were calculated using the FatScan software program (N2system, Osaka, Japan).[16] The patients visited the hospitals to undergo the treatment for obesity and/or metabolic abnormalities such as hypertension, dyslipidemia and type 2 diabetes. Patients with secondary obesity and obesity-related hereditary disorders were excluded from this study. Patients with disease or under treatment that strongly affect the body weight were also excluded. The clinical data were taken at the first visit to the hospital. The clinical characteristics of the subjects are summarized in Table 1. Metabolic syndrome and metabolic abnormalities were diagnosed according to the criteria released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome in April 2005.[4,5] Written informed consent was obtained from each subject, and the protocol was approved by the ethics committee of each institution and by that of Kyoto University.

DNA extraction and single-nucleotide polymorphism genotyping

Genomic DNA was extracted from the blood samples collected from each subject by using Genomix (Talent Srl, Trieste, Italy). We selected 12 single-nucleotide polymorphisms (SNPs) identified as susceptibility loci for obesity by genome-wide association studies in Caucasian populations[11–13] and constructed Invader probes (Third Wave Technologies, Madison, WI, USA) for the following SNPs: rs2815752 in the neuronal growth regulator 1 gene (*NEGR1*); rs10913469 in the SEC16 homolog B gene (*SEC16B*); rs6548238 in the transmembrane protein 18 gene (*TMEM18*); rs7647305 in the ets variant 5 gene (*ETV5*); rs10938397 in the glucosamine-6-phosphate deaminase 2 gene (*GNPDA2*); rs6265 and rs925946 in the brain-derived neurotrophic factor gene (*BDNF*); rs10838738 in the mitochondrial carrier homolog 2 gene (*MTCH2*); rs7498665 in the SH2B adaptor protein 1 gene (*SH2B1*); rs1424233 in the v-maf musculo-aponeurotic fibrosarcoma oncogene homolog gene (*MAF*); and rs29941 and rs11084753 in the potassium channel tetramerisation domain-containing 15 gene (*KCTD15*). The SNPs were genotyped using Invader assays as previously described.[17] The success rate of these assays was >99.0%.

Statistical analysis

For the additive model, we coded the genotypes as 0, 1 or 2 depending on the number of copies of the risk alleles. For the dominant model, homozygosity and heterozygosity with the risk allele were coded as 1 and the other was coded as 0. Multiple linear regression analyses were carried out to test the independent effect of the risk alleles on BMI, VFA and SFA by taking into account the effects of other variables (that is, age and gender) that were assumed to be independent of the effect of each SNP. The Hardy–Weinberg equilibrium was assessed using the χ^2-test.[18] Statistical analysis was carried out using the software R (http://www.r-project.org/). *P*-values were corrected by Bonferroni's adjustment and $P<0.0042$ (0.05/12) was considered statistically significant.

RESULTS

The clinical characteristics and genotypes of the subjects are shown in Tables 1 and 2, respectively. All the SNPs were in the Hardy–Weinberg equilibrium. The BMI, VFA and SFA values for each SNP genotype are represented in Table 3. Multiple linear regression analyses of the anthropometric parameters with respect to the 12 SNPs analyzed are shown in Table 4. No SNPs were not significantly associated with BMI in this population, although a previous study reported that the *SEC16B* rs10913469 and *TMEM18* rs6548238 SNPs were significantly associated with obesity (BMI>30 kg m^{-2}) in the Japanese population.[15]

The *SH2B1* rs7498665 SNP was significantly associated with VFA (P=0.00047) even when the conservative Bonferroni's correction was applied ($P<0.0042$). Previous reports indicate that the rs7498665 SNP is associated with waist circumference[19] or visceral fat mass[20] in the dominant model. The VFA values of the rs7498665 genotype (Table 3) suggest that the dominant model would be the best-fitted model. Therefore, we performed multiple regression analyses by using the

Table 1 Clinical characteristics of the subjects

	Men	*Women*	*Total*
n	556	723	1279
Age (years)	49.4±12.2	52.2±11.3	51.0±11.8
BMI (kg m^{-2})	30.2±6.1	28.1±5.3	29.0±5.8
VFA (cm^2)	155.3±67.7	99.8±53.6	123.9±66.1
SFA (cm^2)	206.7±108.6	241.6±97.2	226.5±103.7
Waist circumference (cm)	97.5±11.3	91.8±10.3	94.2±11.1
Prevalence of metabolic disease (%)			
Dyslipidemia	293 (53)	244 (34)	537 (42)
Hypertension	379 (68)	452 (63)	831 (65)
Impaired fasting glucose	177 (32)	176 (24)	353 (28)
Metabolic syndrome	248 (45)	162 (22)	410 (32)

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; VFA, visceral fat area.
Data are shown as mean±s.d.

Table 2 Genotypic characteristics of the subjects

SNP ID	*Nearby gene*	*Allele 1/2*	*Risk allele*	*Genotype*	*HWE P-value*
rs2815752	*NEGR1*	A/G	A	1113/163/3	0.24
rs10913469	*SEC16B*	T/C	C	690/510/78	0.20
rs6548238	*TMEM18*	T/C	C	6/201/1071	0.29
rs7647305	*ETV5*	T/C	C	201/576/500	0.10
rs10938397	*GNPDA2*	A/G	G	615/537/126	0.58
rs6265	*BDNF*	A/G (Met/Val)	G	207/609/462	0.79
rs925946	*BDNF*	T/G	T	3/100/1175	0.57
rs10838738	*MTCH2*	G/A	G	107/555/616	0.25
rs7498665	*SH2B1*	G/A (Ala/Thr)	G	29/305/945	0.46
rs1424233	*MAF*	A/G	A	726/469/82	0.59
rs29941	*KCTD15*	T/C	C	774/444/60	0.72
rs11084753	*KCTD15*	G/A	G	105/535/638	0.63

Abbreviations: HWE, Hardy–Weinberg equilibrium; SNP, single-nucleotide polymorphism.

Table 3 Mean BMI, VFA and SFA for 12 obesity-risk variants

		Mean ± s.d.								
		BMI ($kg\,m^{-2}$)			VFA (cm^2)			SFA (cm^2)		
SNP ID	Nearby gene	11	12	22	11	12	22	11	12	22
rs2815752	*NEGR1*	29.1 ± 5.9	28.3 ± 5.3	28.5 ± 3.1	124.8 ± 66.7	118.5 ± 62.6	95.2 ± 38.8	226.1 ± 103.1	228.3 ± 108.5	251.0 ± 79.8
rs10913469	*SEC16B*	28.8 ± 5.9	29.2 ± 5.6	29.7 ± 6.5	123.0 ± 66.4	124.9 ± 65.2	124.8 ± 70.4	221.7 ± 103.7	231.6 ± 102.6	234.0 ± 110.5
rs6548238	*TMEM18*	25.9 ± 7.5	29.0 ± 7.2	29.0 ± 5.5	85.9 ± 70.6	123.4 ± 67.1	124.3 ± 65.9	211.0 ± 135.0	222.8 ± 111.3	227.3 ± 102.2
rs7647305	*ETV5*	29.0 ± 5.3	29.1 ± 5.4	29.0 ± 6.3	124.5 ± 66.8	124.5 ± 66.3	123.2 ± 65.8	234.1 ± 99.5	225.6 ± 100.0	224.6 ± 109.6
rs10938397	*GNPDA2*	28.8 ± 5.9	29.1 ± 5.8	29.2 ± 5.3	122.7 ± 68.0	124.5 ± 64.0	127.4 ± 66.3	224.5 ± 103.4	227.9 ± 103.3	229.3 ± 107.5
rs6265	*BDNF*	28.6 ± 5.9	28.7 ± 5.3	29.6 ± 6.3	122.4 ± 68.2	122.9 ± 64.7	126.1 ± 67.1	220.3 ± 92.9	223.5 ± 102.2	233.2 ± 109.9
rs925946	*BDNF*	36.0 ± 10.7	29.5 ± 6.1	28.9 ± 5.7	142.6 ± 11.3	123.3 ± 63.3	124.0 ± 66.4	416.8 ± 155.7	236.3 ± 118.6	225.2 ± 101.9
rs10838738	*MTCH2*	28.7 ± 4.9	29.3 ± 6.4	28.7 ± 5.3	124.5 ± 58.3	125.1 ± 68.5	122.6 ± 65.2	214.1 ± 93.1	233.6 ± 109.6	222.2 ± 99.8
rs7498665	*SH2B1*	29.7 ± 4.8	29.5 ± 6.2	28.8 ± 5.7	134.4 ± 65.3	134.5 ± 70.5	120.2 ± 64.3	231.1 ± 95.9	235.1 ± 98.4	223.6 ± 105.5
rs1424233	*MAF*	29.0 ± 6.0	29.1 ± 5.7	28.4 ± 3.8	123.1 ± 64.7	124.0 ± 65.7	130.6 ± 80.4	222.0 ± 102.6	234.0 ± 109.1	219.7 ± 74.5
rs29941	*KCTD15*	28.8 ± 5.6	29.1 ± 6.0	30.4 ± 6.0	123.9 ± 65.2	122.4 ± 67.3	136.5 ± 68.5	224.8 ± 103.7	228.2 ± 103.5	236.6 ± 106.6
rs11084753	*KCTD15*	29.5 ± 5.7	29.1 ± 5.8	28.9 ± 5.8	128.3 ± 71.9	122.5 ± 64.9	124.5 ± 66.2	233.0 ± 98.6	227.4 ± 102.0	224.7 ± 106.1

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single-nucleotide polymorphism; VFA, visceral fat area.
11, allele1/allele1; 12, allele1/allele2; 22, allele2/allele2. Allele 1 and allele 2 in each SNP is indicated in Table 2.

Table 4 Relationship between obesity loci and adiposity measures

		BMI			VFA			SFA		
SNP ID	Nearby gene	β	s.e.	P-value	β	s.e.	P-value	β	s.e.	P-value
rs2815752	*NEGR1*	0.611	0.448	0.17	7.423	4.847	0.13	−6.293	7.978	0.43
rs10913469	*SEC16B*	0.325	0.255	0.20	2.827	2.753	0.30	4.516	4.532	0.32
rs6548238	*TMEM18*	0.267	0.403	0.51	6.773	4.352	0.12	2.557	7.178	0.72
rs7647305	*ETV5*	0.025	0.221	0.91	1.984	2.386	0.41	2.565	3.929	0.51
rs10938397	*GNPDA2*	0.199	0.236	0.40	0.804	2.547	0.75	4.065	4.190	0.33
rs6265	*BDNF*	0.508	0.223	0.023	1.390	2.410	0.56	6.954	3.968	0.080
rs925946	*BDNF*	0.816	0.545	0.14	0.390	5.897	0.95	18.972	9.685	0.050
rs10838738	*MTCH2*	0.162	0.243	0.51	0.292	2.628	0.91	2.726	4.326	0.53
rs7498665	*SH2B1*	0.536	0.310	0.085	11.717	3.343	0.00047	8.341	5.555	0.13
rs1424233	*MAF*	0.050	0.252	0.84	−2.945	2.722	0.28	−5.311	4.479	0.24
rs29941	*KCTD15*	0.481	0.265	0.070	2.588	2.871	0.37	3.589	4.727	0.45
rs11084753	*KCTD15*	0.332	0.243	0.17	1.562	2.626	0.55	3.242	4.322	0.45

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single-nucleotide polymorphism; VFA, visceral fat area.
Data were derived from a linear regression analysis. BMI, VFA and SFA were adjusted for age and gender.

dominant model and found a significant association between this SNP and VFA (P=0.00022). This association remained significant even after adjusting for age, gender and BMI in the dominant model (P=0.00096). The other SNPs did not show any significant association with VFA. No SNPs, including the *SH2B1* rs7498665, were associated with SFA.

BMI, VFA and SFA are known to be affected by gender; therefore, we compared the anthropometric parameters (BMI, VFA and SFA) among the different genotypes in the men and women (Supplementary Tables 1–3). Association between *SH2B1* rs7498665 SNP and VFA was not significant both in men (P=0.0099) and women (P=0.022). This negative association is most likely due to the decrease in the number of each genotype. The VFA values of the rs7498665 genotype (Supplementary Table 2) suggest that the dominant model would be the best-fitted model both in men and women. By using the dominant model, revealed no significant association between the rs7498665 genotype and VFA in men (P=0.0061) and women (P=0.015).

To confirm the association of the *SH2B1* rs7498665 SNP with VFA, two SNPs (rs4788102 and rs8049439) in linkage disequilibrium of rs7498665 reported by previous study[11] were genotyped (Supplementary Table 4). Both rs4788102 (P=0.00058) and rs8049439 (P=0.0021) SNPs were significantly associated with VFA.

DISCUSSION

In this study, we showed that the *SH2B1* rs7498665 SNP was significantly associated with VFA. Haupt *et al.*[20] used whole-body magnetic resonance imaging to show that this SNP (dominant model) was associated with visceral fat mass. They also reported that the *SH2B1* rs7498665 SNP was not associated with BMI or with non-visceral fat mass. Jamshidi *et al.*[19] reported that the *SH2B1* rs7498665 SNP (dominant model) was associated with waist circumference. Several studies have reported a negative association between the *SH2B1* rs7498665 SNP and abdominal adipose mass (measured using dual energy X-ray absorptiometry)[21] or waist circumference.[22,23]

CT- or magnetic resonance imaging-based analyses are more accurate than waist circumference- and dual energy X-ray absorptiometry-based abdominal fat-mass analysis for evaluating the association between this SNP and visceral fat mass. These data from this study and from the study performed by Haupt *et al.* strongly suggest that the *SH2B1* rs7498665 SNP is associated with visceral fat accumulation.

SH2B1 has four splicing isoforms; that is, α, β, γ and δ, of which SH2-Bβ was originally identified through its association with Janus kinase 2 (JAK2) protein, a cytoplasmic tyrosine kinase that mediates cytokine functions.[24] *SH2B1*-knockout mice have been reported to show severely impaired insulin signaling in the skeletal muscles, liver and adipose tissue, and progressively develop hyperinsulinemia, hyperglycemia and glucose intolerance.[25] *SH2B1*-knockout mice also developed hyperlipidemia, leptin resistance, hyperphagia and obesity.[26] Although data for mesenteric fat have not been reported, both subcutaneous inguinal fat and intra-abdominal (epididymal) fat were found to be increased in *SH2B1*-knockout mice.[26,27] Neuron-specific restoration of *SH2B1* in knockout mice corrected the metabolic disorders, improved leptin regulation of orexigenic neuropeptide expression in the hypothalamus, and protected against high-fat diet-induced leptin resistance and obesity.[27] Ventromedial hypothalamic lesions are reported to induce visceral fat accumulation that does not result in obesity, and to induce hyperglycemia, hyperinsulinemia and hypertriglyceridemia.[28] *SH2B1* was specifically expressed in the brain, including the hypothalamus, in mice with neuron-specific *SH2B1* restoration.[27] Therefore, *SH2B1* expression in hypothalamus (possibly the ventromedial hypothalamic) may have an important role in visceral fat accumulation. As the *SH2B1* rs7498665 SNP is a non-synonymous SNP (G/A, Ala484Thr) and exits in the proline-rich region, the function of the SH2B1 protein might be deteriorated in subjects with the risk G-allele, leading to visceral fat accumulation. The rs4788102 SNP exists in the 5′-flanking region of the *SH2B1* gene, thus, the expression of *SH2B1* may be changed in the subjects with the risk A-allele. It is necessary to investigate whether these SNPs are functional.

In summary, we showed that the *SH2B1* rs7498665 SNP is significantly associated with VFA. This SNP is not associated with BMI or SFA, suggesting that there is a VFA-specific genetic factor. Our results also suggest that the *SH2B1* gene has a role in visceral fat accumulation. However, these results need to be confirmed in other populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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