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HSP 10 is a new autoantigen in both autoimmune pancreatitis and fulminant type 1 diabetes

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ABSTRACT

To search autoantigens in autoimmune pancreatitis (AIP), we have screened the human pancreas cDNA library with a patient's serum and obtained 10 positive clones. Seven out of 10 clones were amylase α-2A, the autoantibody to which was specifically detected in sera from patients with AIP and fulminant type 1 diabetes (FT1DM) [T. Endo, S. Takizawa, S. Tanaka, M. Takahashi, H. Fujii, T. Kamisawa, T. Kobayashi, Amylase α-2A autoantibodies: novel marker of autoimmune pancreatitis and fulminant type 1 diabetes mellitus, Diabetes 58 (2009) 732–737]. Sequencing of 1 out of remaining 3 positive clones revealed that it was identical to heat shock protein 10 (HSP 10) cDNA. Using a recombinant HSP 10, we have developed enzyme-linked immunosorbent assay (ELISA) system for detecting autoantibodies against HSP 10. We found that autoantibody against HSP 10 was also produced with high frequency in sera from patients with AIP (92%) and FT1DM (81%), but not in chronic alcoholic pancreatitis (8%) or healthy volunteers (1.4%). These results suggest that an autoantibody against HSP 10 is also a new diagnostic marker for both AIP and FT1DM.

Autoimmune pancreatitis (AIP), a distinct form of chronic pancreatitis [1], is characterized by (1) irregular narrowing of the main pancreatic duct and swelling of the pancreas, both of which are due to abundant lymphoplasmacytic inflammation [2], (2) increased levels of serum IgG and IgG4, with positive autoantibodies such as anti-lactoferrin antibody (LFAb) or anti-carbonic anhydrase II antibody (CAIIAb) [3,4], and (3) diabetes, which is a frequent complication and can be resolved by corticosteroid treatment [5].

We previously reported that pancreatic islets, as well as exocrine pancreatic cells, were associated with the inflammatory process involving $CD8^+$ and $CD4^+$ T cells, which might induce diabetes mellitus in AIP [6]. These data support the concept that autoimmune mechanisms play pivotal roles in the destruction of endocrine and exocrine pancreatic functions in AIP with diabetes.

Clinically, initial symptoms of AIP include obstructive jaundice and mild abdominal pain, but some patients are asymptomatic, making it difficult to distinguish AIP from idiopathic chronic pancreatitis or cancer of the pancreas. In such cases, detection of autoantibodies is an important means for diagnosing AIP; however, some proportion of patients with AIP are negative for LFAb and CAIIAb [3,4].

We encountered an AIP patient whose serum IgG and IgG4 levels were 3498 mg/dl and 2430 mg/dl, respectively. High concentrations of IgG in this case prompted us to search for new autoantigens primarily associated with AIP. We have screened λTriplEx2 human pancreas cDNA library with the patient's serum and obtained 10 positive clones. Seven out of 10 clones were identical to amylase α-2A (AMY) [7].

In this report, we further analyzed remaining positive clones other than AMY. Sequencing of 1 out of 3 clones revealed that it was identical to heat shock protein 10 (HSP 10) cDNA. We determined frequency of autoantibody against HSP 10 in AIP and other pancreatic diseases.

Materials and methods

Subjects. Serum used for screening the human pancreas cDNA library was obtained from a 67-year-old male patient with AIP (A.O.), whose detail laboratory data were described previously [7]. Additional 19 AIP sera, 24 sera from patients with chronic alcoholic pancreatitis, 24 serum from patients with pancreas tumor [cancer ($n = 10$) and intraductal papillary mucinous tumor (IPMT, $n = 14$)] were recruited. Sera from FT1DM ($n = 16$, 11 cases at the onset and 5 cases after onset) was diagnosed by criteria (fasting C-peptide $\leqslant 0.033$ nmol/L and HbA_{1c} is $\leqslant 8.0\%$ or $\sum$C-peptide $\leqslant 0.540$ nmol/L and HbA_{1c} is $\leqslant 8.0\%$), type 2 diabetes (T2DM) ($n = 50$), Hashimoto's thyroiditis ($n = 54$) and control sera (healthy

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Table 1
Clinical characteristics of the subjects.

Type of diabetes	N	Age (Y)	Sex (M/F)	Duration[a] of diabetes month (M)/day (D)	Treatment by insulin (N)
Autoimmune pancreatitis	20	67.1 (9.8)	18 / 2	–	12
At the onset[a] and before corticosteroid treatment	12				
After corticosteroid treatment	8				
Chronic alcoholic pancreatitis	24	62.8 (11.7)	18 / 6	–	–
Pancreatic tumor	24	69.0 (10.1)	10 / 14	–	–
Cancer	10				
IPMT	14				
Fulminant type 1 diabetes	16	41.6 (14.6)	11 / 5	4.7 M (8.4)	16
At the onset[b]	11			23 M (27)	
After the onset	5			406 D (343)	
Acute onset type 1 diabetes	40	24.9 (16.2)	13 / 27	28.8 M (45.0)	40
At the onset[b]	18			0.7 M (0.9)	
After the onset	22			51.8 M (50.3)	
Type 2 diabetes	50	62.2 (12.7)	35 / 15	138.5 M (100.3)	27
Normal	71	40.7 (21.5)	39 / 32	–	–
Hashimoto's thyroiditis	54	57 (12.1)	6/48	–	–

Mean (SD), IPMT; intraductal papillary mucinous tumor.
[a] Duration; from onset of diabetes to time of sample collection.
[b] At onset; within 3 months after onset.

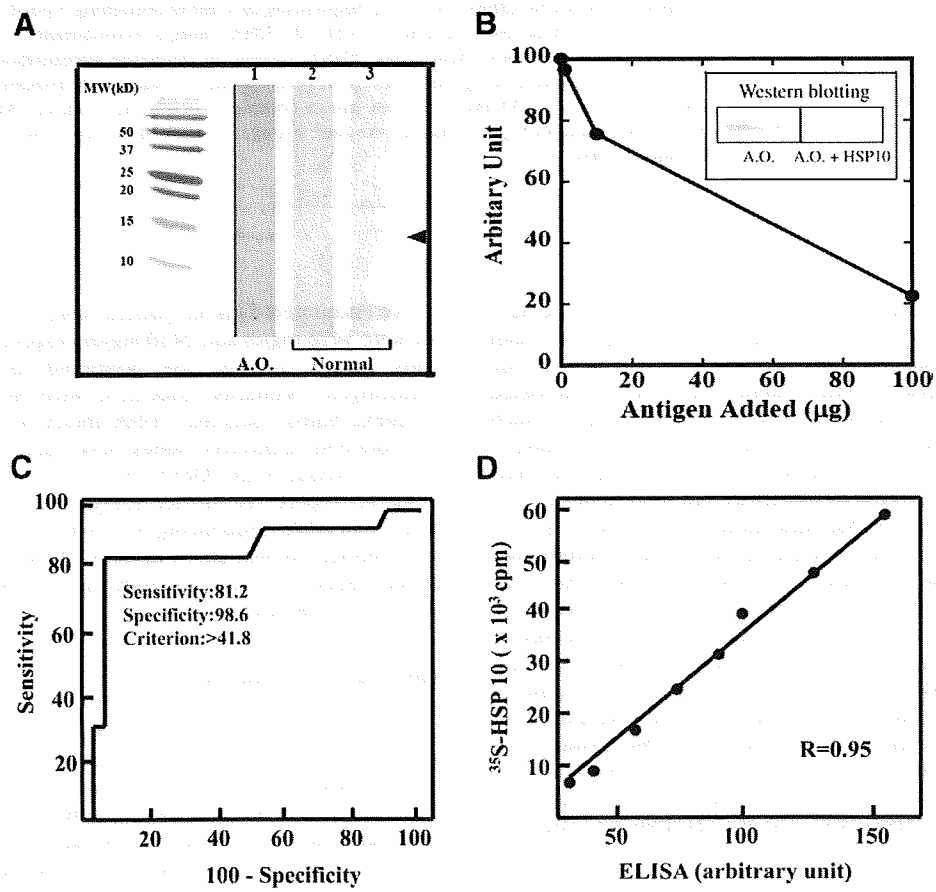

Fig. 1. Western blot analysis and development of an ELISA system for detecting HSP 10 autoantibody. (A) Western blot analysis. Recombinant human HSP 10 was electrophoresed in a 0.1% SDS–15% polyacrylamide gel and transferred onto a PVDF membrane. The membrane was reacted with serum (1000×) from a patient with AIP (lane 1) and normal control sera (lanes 2 and 3). (B) By coating the plate with the recombinant protein, we developed an ELISA system for detecting anti-HSP 10. One milliliters of the patient's serum (1:1000) was preincubated with 1, 10, and 100 mg/ml of the recombinant protein overnight at 4 °C and then added to the plate and incubated for 1 h at 37 °C. The data were the mean of triplicate assays. The inset shows that the positive reaction in Western blotting was reduced when serum from a patient with AIP (A.O.) was preincubated with 100 mg of the recombinant protein. (C) ROC analysis. We carried out ROC analysis of the healthy normal volunteers ($n = 71$) and fulminant type 1 diabetes patients ($n = 16$) with MedCalc. (D) Correlation between the result of ELISA and that of immunoprecipitation. Sera from 11 patients with AIP (●) were assayed by ELISA and IP for detecting the autoantibody against HSP 10.

volunteer, $n = 71$ (39 male and 32 female), were also recruited from our cohort (Table 1). These patients' sera were almost overlapped with our previous report [7], in which detail diagnostic criteria for each disease and patients' profiles were described.

Immunoscreening. λTriplEx2 human pancreas large insert cDNA library (HL5517u) was screened with a serum from AIP patient (A.O.) as described previously [7].

Preparation of the recombinant human heat shock protein 10 (HSP 10). A cDNA fragment of the positive clone was amplified by polymerase chain reaction (PCR) with the sense primer 5′-ATGGGGA TCCGCAGGACAAGCGTTTAGA-3′ and anti-sense primer 5′-CTTCG AATTCTCAGTCTACGTACTTTCC-3′. The PCR product was digested with BamHI and EcoRI, and then ligated into pTrcHisB (Invitrogen Co., Carlsbad, CA). After sequencing, the plasmid was transfected into *Escherichia coli* BL21 (Novagen, Darmstadt, Germany). The production of the recombinant protein was induced with 1 mM IPTG, and purified by HisBond® column chromatography (Invitrogen Co., Carlsbad, CA).

Western blot analysis. The 0.1% SDS–15% polyacrylamide gel electrophoresis and transference of the proteins onto the polyvinylidene difluoride (PVDF) membrane were carried out as described previously [8]. The membrane was reacted with goat horseradish peroxidase-conjugated anti-human IgG (1:2000) for 30 min at room temperature. Positive reaction was detected by the same way as described in the section on immunoscreening.

Enzyme-linked immunosorbent assay (ELISA) for detecting autoantibody against human HSP 10. Autoantibody against human HSP 10 was measured by ELISA using the methods as described previously [5,7]. The bound antibody was specifically reacted with goat horseradish peroxidase-conjugated anti-human IgG (1:2000) in 1% BSA for 30 min at room temperature. After washing, the plate was incubated with 100 ml of 1-Step Slow TMB-ELISA (PIERCE, Rockford, IL) for 30 min. The reaction was terminated by adding 100 ml of 1 M H_2SO_4. Intra-assay CV was 1.6% and inter-assay CV was 8.8%.

In vitro translation and immunoprecipitation assay. cDNA fragment of HSP 10 was amplified by PCR, and then ligated into pcDNA3.1. ^{35}S labeled human HSP 10 was prepared with PROTEIN script II (Ambion, Austin, TX) and [^{35}S] methionine (GE Healthcare, Piscataway, NJ). ^{35}S labeled HSP 10 was incubated with patients' sera (100×) in 100 μl of phosphate buffer saline (PBS) containing 1% bovine serum albumin at 4 °C overnight. Bound antigens were catched, washed and released from the column using Catch and Release v2 immunoprecipitation system (MILLIPOR, Temecula, CA).

Ethics. An ethics committee approved all study protocols, and patients and first-degree relatives of patients with FT1DM gave informed consent.

Statistical analysis. Statistical analysis was carried out using Fisher's exact test (JMP, Cary, NC), in which we considered statistically significant if P values were <0.05. ROC analysis was carried out with MedCalc (MedCalc Software, Mariakerk, Belgium).

Results

Cloning of HSP 10 cDNA from human pancreas cDNA library

We screened λTriplEx2 human pancreas cDNA library with the serum from the patient with AIP (A.O.) and obtained 10 positive clones. Of 10 clones, 7 were AMY cDN [7], but remaining 3 clones did not cross-hybridized with ^{32}P-AMY cDNA. Insert size of 1 (clone 34) out of 3 clones is 800 bp, and sequencing of it revealed that the clone was identical to human HSP 10. When compared to the nucleotide sequence of the human HSP 10 cloned by Monzini et al. [8], the clone contained the full coding sequence, the 5′ end of which started from −75 bp (A in ATG is designated as +1) and the 3′ end was +724 bp.

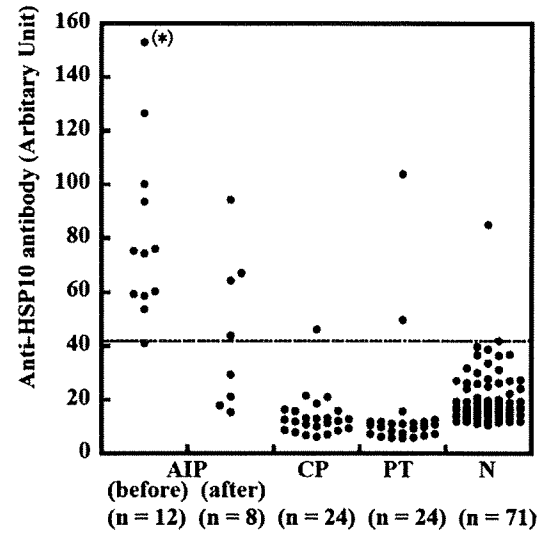

Fig. 2. Prevalence of autoantibody against human HSP 10 in patients with various pancreatic diseases. The prevalence of autoantibody against HSP 10 in patients with autoimmune pancreatitis (AIP; before treatment with corticosteroid, $n = 12$; after treatment with corticosteroid, $n = 8$), chronic alcoholic pancreatitis (CP, $n = 24$), pancreatic tumor (PT, $n = 24$), and normal controls (healthy volunteers N; $n = 71$) was studied by ELISA as described in Materials and methods. Cut-off value is shown by dotted line. The data were the mean of triplicate assays. (*): $p < 0.001$ by Fisher's exact test.

Western blot analysis, ELISA system and immunoprecipitation assay for detecting HSP 10 autoantibody

We produced recombinant human HSP 10 in *E. coli* BL21 and carried out Western blot analysis. The patient's serum (A.O.) clearly recognized the 14 kDa recombinant protein (lane 1), but sera from healthy volunteers (lanes 2 and 3) did not (Fig. 1A). When the patient's serum was preincubated with the recombinant protein, positive staining was abolished (Fig. 1B, inset), suggesting that the autoantibody reacted with the recombinant protein.

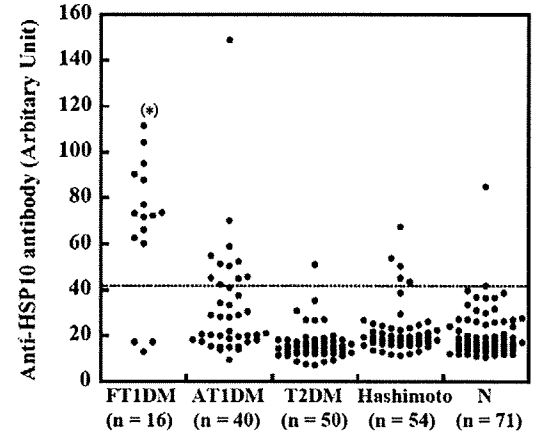

Fig. 3. Prevalence of autoantibody against HSP 10 in patients with various types of diabetes. The prevalence of autoantibody against HSP 10 in patients with fulminant type 1 diabetes (FT1DM, $n = 16$), acute onset type 1 diabetes (AT1DM, $n = 40$), type 2 diabetes (T2DM, $n = 50$), normal controls (N, $n = 71$) and patients with Hashimoto's thyroiditis (Hashimoto, $n = 54$) was studied by ELISA as described in Materials and methods. The data were the mean of triplicate assays. The dotted line is the cut-off value. (*): $p < 0.001$ by Fisher's exact test.

Next, by coating the protein onto the plate, we developed an ELISA system for detecting anti-HSP 10 antibodies in the serum. When compared to the normal serum, patient serum showed a strong signal. This reaction was absorbed when the patient's serum was preincubated with recombinant HSP 10 protein (Fig. 1B). To obtain a cut-off value for positivity, we carried out ROC analysis of the control (healthy volunteers ($n = 71$)) and FT1DM patients ($n = 16$) with MedCalc software. Analysis of the criterion values and coordinates of the curve indicated that, at value 41.8, sensitivity, specificity, positive predictive value and negative predictive value were 81.25%, 98.59%, 92.85% and 99.87%, respectively (Fig. 2C) (area under the ROC curve: 0.88, significance level P: 0.0001). So, we set 41.8 as cut-off value for positivity.

We further prepared ^{35}S labeled HSP 10 by in vitro transcription and translation, and then immunoprecipitation assay was carried out using IgGs from the patients with AIP. The amounts of precipitated ^{35}S-HSP 10 were well correlated with the ELISA signals (Fig. 1D).

Prevalence of autoantibody against human HSP 10 in patients with AIP

Using the ELISA system, we determined the prevalence of autoantibody against HSP 10. Of the patients with AIP ($n = 12$) who were newly diagnosed but not yet treated with corticosteroid, 92% were positive for HSP 10 autoantibodies ($p < 0.0001$, Fisher's exact test). When 8 out of these 12 patients with AIP were treated with corticosteroid, 4 patients (63%) became to be negative for the autoantibody. Only 2 (8%) were positive in sera from 24 patients with chronic alcoholic pancreatitis, and 2 (8%) were positive in sera from 24 patients with a pancreas tumor (pancreatic cancer, $n = 10$; IPMT, $n = 14$) (Fig. 2).

Prevalence of autoantibody against human HSP 10 in the patients with fulminant type 1 diabetes, acute onset type 1 diabetes and type 2 diabetes

Interestingly, of the 16 patients in whom FT1DM was newly diagnosed, 13 (81%) were positive ($p < 0.0001$, Fisher's exact test) for the HSP 10 autoantibody, with titers nearly comparable to those of patients with AIP (Fig. 3). The autoantibody was detected with low frequency in patients with AT1DM (29%), and only 1 in the patients with T2DM (2%). Antibodies were detected in 9% of patients with Hashimoto's thyroiditis, a representative organ-specific autoimmune disease.

Longitudinal changes of HSP 10 autoantibodies in patients with AIP or fulminant type 1 diabetes

The levels of HSP 10 autoantibodies were measured by ELISA in the patients with AIP ($n = 2$), who were followed up to 12–14 months, and in the patients with FT1DM ($n = 2$), who were followed up to 7–10 weeks immediately after the clinical onset. HSP 10 autoantibodies from 2 patients with AIP were positive at onset and were sustained until the initiation of corticosteroid treatment, while the titer of the autoantibodies decreased or disappeared after

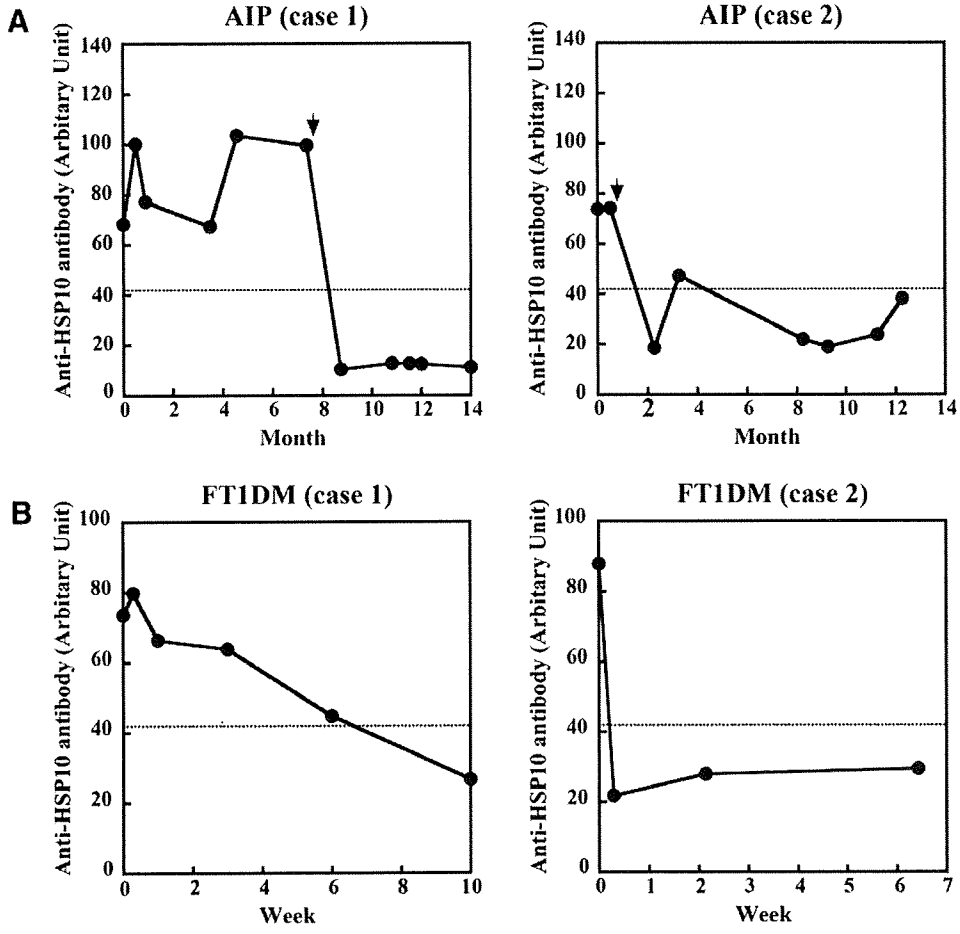

Fig. 4. Longitudinal changes of HSP 10 autoantibodies in patients with AIP or fulminant type 1 diabetes. (A) Time course of HSP 10 autoantibodies in two patients with AIP. The titers of HSP 10 autoantibodies from Case 1 and Case 2 are shown. Each value is the mean of triplicate assays. Arrows indicate the initiation point of corticosteroid treatment in each case. The dotted line shows the cut-off value. (B) Time course of HSP 10 autoantibodies in two patients with FT1DM. The titers of HSP 10 autoantibodies from Case 1 and Case 2 are shown. Each value is the mean of triplicate assay.

initiation of corticosteroid treatment (Fig. 4A) in parallel with decline of serum IgG concentration (data not shown). HSP 10 autoantibodies from two patients with FT1DM were also positive at onset and the titer decreased with the duration of diabetes (Fig. 4B).

Discussion

In the present study, we detected a new autoantibody against HSP 10 in patients with active AIP, but not in patients with chronic alcoholic pancreatitis or pancreatic tumors. Titers of the autoantibody were high at onset, and rapidly decreased in response to corticosteroid treatment, suggesting that HSP 10 is a new diagnostic and clinical marker for AIP.

It is particularly interesting that HSP 10 autoantibodies are detected in a high proportion of the patients with newly diagnosed FT1DM and AT1DM. AMY autoantibodies were also detected with similar prevalence both in patients with AIP and FT1DM [7], suggesting that both diseases are closely related with each other.

At present, the pathogenesis of FT1DM is associated with autoimmunity is still unknown [9], but we have demonstrated $CD4^+$ and $CD8^+$ T cell infiltration into pancreatic exocrine cells as well as the islets, which decreased immediately after the onset of FT1DM [10]. These results, as well as the presence of an autoantibody against HSP 10, suggest that the disease might be autoimmune-related, involving the exocrine and the endocrine pancreas functions. Our data also suggest that the measurement of the autoantibodies against HSP 10 is useful for diagnosing FT1DM, which sometimes causes serious diabetic ketoacidosis and is life-threatening for affected individuals who may be pregnant [11].

HSP 10 and HSP 60 form mitochondrial chaperoning complexes and are believed to play a role in the maintenance of normal mitochondrial function. However, overexpression of these proteins during cellular stress makes them an important part of immune system recognition. Following their release from inflamed or necrotizing tissues, they may be recognized by the cell surface receptors of the host immune systems [12].

Recently, it has been proposed that HSP 60, a counterpart of HSP 10, is an early antigen in the triggering of insulin-dependent diabetes mellitus; as the presence of an antibody to HSP 60 precedes the disease, HSP-reactive T cells can transfer the disease to prediabetic NOD mice, and vaccination with the protein blocks disease induction [13,14]. Furthermore, studies using HSP 60 transgenic mice indicated that it is involved in the islet-cell destruction that occurs in NOD mice [15].

With respect to HSP 10, immunohistochemical studies revealed the presence of HPS 10 in the pancreas, especially in acinal cells and also islet cells [16]. It is also reported that HSP 10 is detected outside the cells [17]. So, possibility exists that positive HSP 10 antibody in AIP and FT1DM may reflect only pancreatic destruction.

However, recent findings have revealed that extracellular HSP 10 interacts with Toll-like receptor 4 and inhibits the induction of nuclear factor-κB, followed by the reduction of serum tumor necrosis factor and RANTES (regulated upon activation, normal T cell expressed and secreted) levels and the elevation of serum interleukin-10 levels [18]. Therefore, HSP 10 itself has anti-inflammatory properties or has a role in the modulation of innate immune response. Indeed, administration of HSP 10 to the patients with rheumatoid arthritis reduces the signs and symptoms of the disease [19]. So, we speculate that HSP 10, like HSP 60, might be involved in the pathogenesis of both diseases.

Taking into account these observations, further studies are needed to clarify the roles of HSP 10 or its autoantibody in the development of both AIP and FT1DM.

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Amylase α-2A Autoantibodies

Novel Marker of Autoimmune Pancreatitis and Fulminant Type 1 Diabetes

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OBJECTIVE—The pathogenesis of autoimmune pancreatitis (AIP) and fulminant type 1 diabetes remains unclear, although it is known that immune-mediated processes severely compromise the endocrine and exocrine functions in both diseases.

RESEARCH DESIGN AND METHODS—We have screened a λTriplEx2 human pancreas cDNA library with serum from a patient with AIP and obtained positive clones. Sequence analysis revealed that 7 of 10 clones were identical to human amylase α-2A. Using a recombinant COOH-terminal amylase α-2A protein, we developed an enzyme-linked immunosorbent assay system to detect autoantibodies against human amylase α-2A.

RESULTS—All 15 serum samples from patients with AIP recognized the recombinant protein, whereas sera from 25 patients with chronic alcoholic pancreatitis and sera from 25 patients with a pancreas tumor did not. Interestingly, 88% (15/17) of patients with fulminant type 1 diabetes were positive for an autoantibody against amylase α-2A. These antibodies were detected in 21% of patients with acute-onset type 1 diabetes (9 of 42) and 6% of type 2 diabetic patients (4 of 67).

CONCLUSIONS—These results suggest that an autoantibody against amylase α-2A is a novel diagnostic marker for both AIP and fulminant type 1 diabetes and that, clinically and immunologically, AIP and fulminant type 1 diabetes are closely related.
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Recently, autoimmune pancreatitis (AIP), a unique form of chronic pancreatitis, has been reported as a discrete disease entity (1). It is characterized by *1*) irregular narrowing of the main pancreatic duct and swelling of the pancreas, both of which are due to abundant lymphoplasmacytic inflammation to the exocrine pancreas (2); *2*) the increased serum level of IgG and IgG4; *3*) positive autoantibodies such as lactoferrin autoantibody or carbonic anhydrase II (CAII) autoantibody (3,4); and *4*) a high prevalence of diabetes with complications (5).

We recently reported that a high proportion of pancreatic islets and exocrine pancreatic tissues were infiltrated by $CD4^+$ or $CD8^+$ T-cells in the inflammatory process, which might induce diabetes in AIP (6). In addition, treatment with prednisolone improved insulin secretion and glycemic control in AIP patients (5). These data support the concept that autoimmune mechanism(s) plays a pivotal role in the destruction of the endocrine and exocrine pancreas in AIP patients with diabetes.

Clinically, the most common initial symptom of AIP is jaundice, but in some patients, no symptoms or only mild symptoms, frequently without acute attacks of pancreatitis, may be present (7). It is difficult to distinguish AIP from other types of chronic pancreatitis or cancer of the pancreatic head (8). In such cases, detection of autoantibodies is useful for diagnosing AIP, but a proportion of patients with AIP are negative for autoantibodies against lactoferrin and CAII (3,4).

We encountered an AIP patient whose serum IgG and IgG4 levels were 3,498 and 2,430 mg/dl, respectively. It has been reported that median levels (5th and 95th percentiles) of IgG and IgG4 from patients with AIP were 2,389 mg/dl (1,349 and 4,310) and 742 mg/dl (265 and 1,150), respectively (3), so high concentrations of IgG in this case prompted us to search for new autoantigens associated with AIP. We also searched for the presence or absence of new autoantibodies in patients with abrupt onset and severe ketoacidosis-prone type 1 diabetes [called fulminant type 1 diabetes (9,10)], which involve the exocrine pancreas and the endocrine pancreas.

RESEARCH DESIGN AND METHODS

Serum used for screening the human pancreas cDNA library was obtained from a 67-year-old male patient (A.O.), who was admitted to our hospital complaining of slight abdominal pain and jaundice. Computed tomography revealed an enlarged pancreas, and laboratory findings showed high concentrations of IgG and IgG4. Tests for anti-lactoferrin and anti-CAII antibodies were both positive, but those for anti-nuclear antibody, anti-mitochondrial antibody, and rheumatoid factor were negative.

Additional AIP sera were obtained from 14 newly diagnosed patients at the University of Yamanashi Hospital and Toranomon Hospital, Tokyo. Diagnosis of AIP was based on criteria proposed by the Japan Pancreas Society (11). Our 15 patients filled criterion 1 (narrowing of the main pancreatic duct or enlargement of pancreas by imaging studies), together with criterion 2 (high serum γ-globulin, IgG, or IgG4 or the presence of autoantibodies, such as anti-nuclear antibodies and rheumatoid factor) and/or criterion 3 (marked interlobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area). Serum samples were taken from 25 patients with chronic alcoholic pancreatitis, who were diagnosed according to a history of alcohol abuse, impaired exocrine pancreatic function, and the presence of calcified precipitates in the pancreas by imaging studies [Japan Pancreas

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TABLE 1
Clinical characteristics of subjects

Type of diabetes	*n*	Age (years)	Sex (male/female)	Duration of diabetes (months)*	Treatment by insulin*
Autoimmune pancreatitis	15	66 (58–75)	14/1	—	8
Before PSL	12				
After PSL	3				
Chronic alcoholic pancreatitis	25	63 (53–70)	18/7	—	10
Pancreatic tumor	25	71 (63–73)	12/13	—	8
Cancer	8				
IPMT	17				
Fulminant type 1 diabetes	17	40 (28–53)	14/3		17
At onset†	13			0.76 ± 0.20	
After onset	4			13.5 ± 2.38	
Acute-onset type 1 diabetes	42	25 (23–33)	14/28	29.0 ± 45.0	42
At onset†	22			0.7 ± 0.9	
After onset	20			51.0 ± 50.0	
Type 2 diabetes	67	62 (58–65)	43/24	130 ± 91.0	37
Hashimoto's thyroiditis	47	60 (55–62)	6/41	—	—
Control subjects	100	47 (40–48)	59/41	—	—

Data are medians (95% CI) or means ± SD. PSL, prednisolone; IPMT, intraductal papillary mucinous tumor. *Duration from the onset of diabetes to the time of sample collection. †At onset; within 3 months after onset.

Society, criteria for chronic pancreatitis 2001 (12)]. Twenty-five serum samples were recruited from patients with pancreas tumor (cancer [n = 8] and intraductal papillary mucinous tumor [IPMT, n = 17]). Fulminant type 1 diabetes (n = 17, 13 cases at the onset and 4 cases after onset) was diagnosed by criteria (fasting C-peptide ≤0.033 nmol/l and A1C ≤8.0% or ∑C-peptide ≤0.540 nmol/l and A1C ≤8.0%) as reported previously (13,14). Fulminant type 1 diabetes associated with pregnancy (15) was excluded from the present study. Acute-onset type 1 diabetes (n = 42) (12) and type 2 diabetes (n = 67) samples were also recruited. The patients' clinical characteristics are summarized in Table 1. Serum from patients with Hashimoto's thyroiditis (n = 47) were also studied. Diagnosis of the disease was made by elastic goiter and autoantibodies against both thyroglobulin and thyroid peroxidase. Control sera were obtained from 100 (59 male and 41 female) healthy volunteers.

Immunoscreening. The λTriplEx2 human pancreas large insert cDNA library (HL5517u) and *Escherichia coli* XL-1 competent cells were obtained from BD Biosciences Clontech (Palo Alto, CA). The plaques on the plate were transferred to nitrocellulose filters presoaked with 10 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG), washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), and blocked with TBST containing 1% BSA. The filters were incubated overnight at 4°C with the sera from the patient with AIP (A.O.) at a dilution of 1:500. After washing four times with TBST, the filters then reacted with goat horseradish peroxidase–conjugated anti-human IgG (American Qualex, San Clemente, CA) at a dilution of 1:2,000 for 30 min at room temperature. The filters were also washed four times with TBST; positive reaction was detected with 3,3′-diaminobenzidine.

Preparation of the recombinant human AMY-2A. A cDNA fragment of the positive clone was amplified by PCR with the sense primer, 5′-ATGGGGATC CTTGGGGTTTCGTACCTTCTGACAGA, and antisense primer, 5′-CTTCGAAT TCCCAATTTAGATTCAGCATGAATTGC. The PCR product was digested with *Bam*HI and *Eco*RI and then ligated into pTrc His B (Invitrogen, Carlsbad, CA). After sequencing, the plasmid was transfected into *E. coli* BL-21 (Novagen, Darmstadt, Germany). The production of the recombinant protein was inducted with 1 mmol/l IPTG and purified by His Bond column chromatography.

Western blot analysis. The 0.1% SDS–15% PAGE and transferring onto the nitrocellulose membrane was carried out as previously described (16) with slight modifications as follows: The membrane was blocked with 5% skim milk and 5% goat serum in TBS and then incubated with sera from the patients with AIP (1:500) overnight at 4°C. After washing five times with TBST, the membrane was reacted with goat horseradish peroxidase–conjugated anti-human IgG (1:2,000) for 30 min at room temperature. Positive reaction was detected by the same way as described in IMMUNOSCREENING.

In vitro translation and immunoprecipitation. A cDNA fragment of AMY-2A was amplified by PCR with the sense primer, 5′-ATGGGGATCCATG TGGGGTTTCGTACCTTCTGACAGA, and antisense primer, 5′-CTTCGAATTC CCAATTTAGATTCAGCATGAATTGC, which added an ATG codon at the NH_2-terminus. The PCR product was digested with *Bam*HI and *Eco*RI and then ligated into pcDNA3.1. ^{35}S-labeled human AMY-2A was prepared with PROTEIN script II (Ambion, Austin, TX) and [^{35}S]methionine (GE Healthcare, Piscataway, NJ). ^{35}S–AMY-2A was incubated with patients' sera (×100) or anti-human amylase antibody (×100; sc-12821; Santa Cruz Biotechnology, Santa Cruz, CA) in 200 μl PBS containing 1% BSA at 4°C overnight, with 10 μl GammaBind G Sepharose (GE Healthcare) added. After further incubation at room temperature for 60 min, the mixtures were centrifuged at 10,000 rpm for 5 min. The pellets were washed three times with PBS containing 0.05% Tween 20 (PBST). Final pellets were directly counted or dissolved with 10 mmol/l Tris-HCl (pH. 6.8) containing 0.1% SDS, boiled for 3 min, and loaded onto a 0.1% SDS–15% polyacrylamide gel.

Enzyme-linked immunosorbent assay for detecting autoantibody against human AMY-2A. Autoantibody against human AMY-2A was measured by enzyme-linked immunosorbent assay (ELISA) using methods previously described (5). In brief, a microtiter plate (Coster 3590; Corning, Horseheads, NY) was coated with 50 μl 0.1 μg recombinant human AMY-2A overnight at 4°C. After washing the plate three times with PBST, the plate was incubated with 200 μl 1% BSA in PBS for 30 min. Next, the patients' sera were tested in triplicate at dilutions of 1:200 in 1% BSA for 1 h. The bound antibody was specially reacted with goat horseradish peroxidase–conjugated anti-human IgG (1:2,000) in 1% BSA for 30 min at room temperature. After washing, the plate was incubated with 100 μl 1-Step Slow TMB-ELISA (Pierce, Rockford, IL) for 30 min. The reaction was terminated by adding 100 μl 1 mol/l H_2SO_4, and absorbance was determined at an optical density of 450 nm. Intra- and interassay coefficient of variation, determined with the same lot of five ELISA plates, were 4.28 and 7.72%, respectively.

Ethics. An ethical committee approved all study protocols, and patients gave informed consent.

Statistical analysis. Statistical analysis was carried out using Fisher's exact test (JMP, Cary, NC), in which we considered statistically significant if P values were <0.05. Receiver operating characteristic (ROC) analysis was carried out with MedCalc (MedCalc Software, Mariakerke, Belgium).

RESULTS

Cloning of cDNAs from human pancreas. We completely screened 2 × 10^4 plaques with the AIP patient's serum (A.O.) and obtained 10 positive clones. Nucleotide sequencing of the insert cDNAs and a subsequent homology search revealed that 7 of 10 clones were identical to human amylase-2A (AMY-2A). When compared with the nucleotide sequence of the human AMY-2A cloned by Wise et al. (17), four of seven clones contained the full coding sequence, whereas the 5′ ends of the other three clones started from 61, 799, and 897 bp (A in ATG is designated as 1) (Fig. 1). Other nonamylase clones were those of the housekeeping genes, such as the heat shock protein and the nuclear protein.

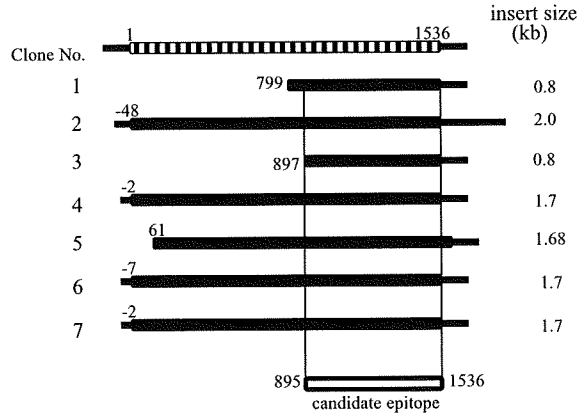

FIG. 1. Cloning of human amylase α-2A cDNAs from λTriplEx2 human pancreas cDNA library. Seven clones of human amylase α-2A cDNAs. Their lengths and 5′-ends are shown (A in ATG is designated as 1). The top bar indicates human amylase α-2A cDNA as reported by Wise et al. (17), and the common regions shared by all seven clones, from codons 299 to 512, are shown in the bottom bar.

Western blot analysis, immunoprecipitation, and ELISA system for detecting anti-human AMY-2A. Because IgG from the AIP patient used for screening recognized four different lengths of human AMY-2A clones, we hypothesized that the regions shared by these four clones, from codons 299 to 512, might contain a common epitope for the patient's IgG (Fig. 1). Therefore, we produced histidine-tagged human AMY-2A from codons 299 to 512 (AMY-2A/299–512) in *E. coli* BL21 and carried out Western blot analysis (Fig. 2*A*). Patient's serum (A.O.) recognized the 30-kDa recombinant protein (*line 1*), but sera from healthy volunteers did not (*lines 3* and *4*). When the patient's serum was preincubated with the recombinant protein, positive staining was abolished (*line 2*), suggesting that the autoantibody reacted with the recombinant protein, which contains the epitope.

Anti-human AMY-2A antibody produced in goat was bound to the in vitro–translated ^{35}S–AMY-2A and was precipitated by protein G–sepharose (Fig. 2*B*). IgG from two patients with AIP also bound to the labeled protein and was precipitated, but the IgG from two healthy volunteers did not (Fig. 2*B*). This recombinant fluid phase autoantibody assay with in vitro transcription and translation of AMY-2A without additional amino acids, such as His-Tag, confirmed the specificity of the autoantibody against the protein.

Next, by coating the protein onto the plate, we developed an ELISA system for detecting anti-amylase antibodies in the serum. When compared with the normal serum, patient sera showed strong signals, which were well correlated with immunoprecipitated ^{35}S–AMY-2A by protein G–sepharose (Fig. 2*C*). This positive reaction in ELISA was displaced in a concentration-dependent fashion by AMY-2A/299–512 (Fig. 2*D*). When the AIP patient's serum (A.O.) was diluted, we could detect positive signals up to ×1,000 dilution (Fig. 2*E*). To obtain a cutoff value for positivity, we carried out ROC analysis of the healthy

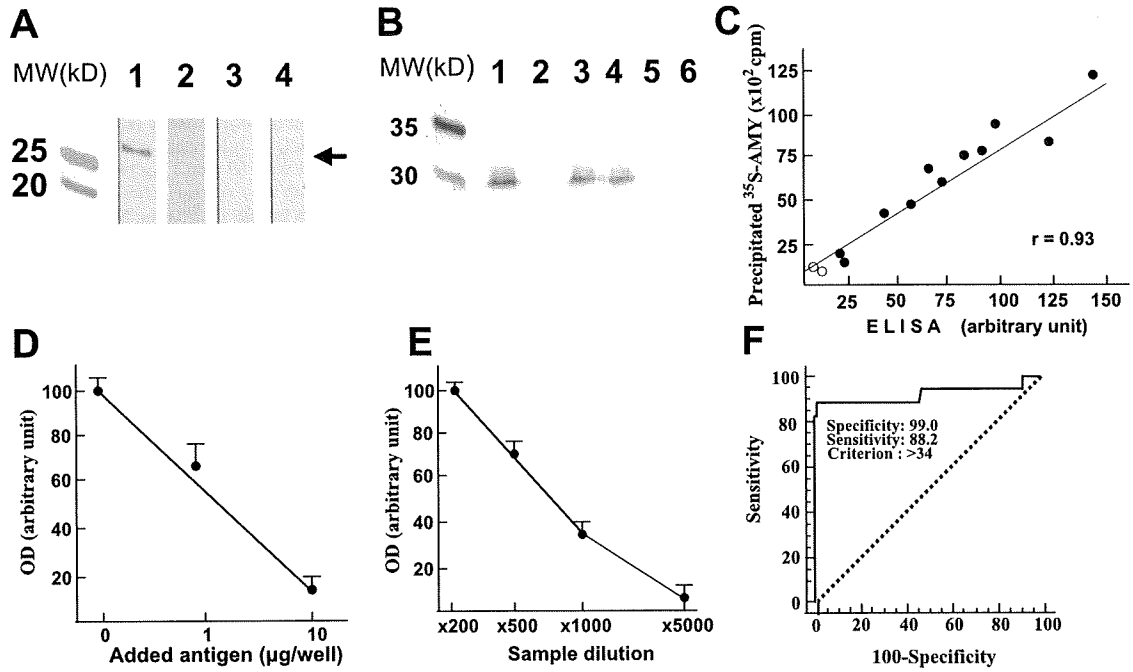

FIG. 2. Western blot analysis and ELISA for detecting anti-human AMY-2A. *A*: Western blot analysis. Recombinant human AMY-2A (50 ng) from codons 299 to 512 (AMY-2A/299–512) was electrophoresed in 0.1% SDS–15% polyacrylamide and transferred onto a nitrocellulose filter. The filters reacted with serum (×1,000) from an AIP patient (*line 1*) and normal control sera (*lines 3* and *4*). Line 2, AIP patient's serum preincubated with 1 μg/ml AMY-2A/299–512. MW, molecular weight markers. *B*: Immunoprecipitation of ^{35}S–AMY-2A with antibodies. ^{35}S–AMY-2A was incubated with goat anti-amylase (*line 1*), normal goat IgG (*line 2*), sera from AIP patients (*lines 3* and *4*), and sera from healthy volunteers (*lines 5* and *6*) and then precipitated with protein G–sepharose. The pellets were electrophoresed in 0.1% SDS–15% polyacrylamide and analyzed with Bas 2000 image analyzer (Fujix, Tokyo). *C*: Correlation between the result of ELISA and that of immunoprecipitation. By coating the recombinant human AMY-2A/299–512, we developed an ELISA system for detecting anti-human AMY-2A. Sera from 11 patients with AIP (●) and two normal control subjects (○) were assayed by ELISA and immunoprecipitation for detecting the autoantibody. *D*: Absorption of positive ELISA signal with recombinant AMY-2A. One milliliter of a patient's serum (1:500) was preincubated with the recombinant protein at the indicated dose overnight at 4°C, and then the serum was used as the first antibody. The data are the mean of triplicate values. OD, optical density. *E*: Serum dilution experiment in ELISA assay. Positive serum from patient A.O. was diluted as indicated, and ELISA assay was carried out. The data are the mean of triplicate values. *F*: ROC analysis of the healthy volunteers and fulminant type 1 diabetic patients. We carried out ROC analysis of the healthy volunteers (n = 100) and fulminant type 1 diabetic patients (n = 17) with MedCalc.

TABLE 2
Criterion values and coordinates of the ROC curve

Criterion	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value
≥3.2	100.00 (80.3–100.0)	0.00 (0.0–3.7)	14.5	
>11.4	100.00 (80.3–100.0)	9.00 (4.2–16.4)	15.7	100
>11.8	94.12 (71.2–99.0)	9.00 (4.2–16.4)	15.0	90.0
>17.3	94.12 (71.2–99.0)	53.00 (42.8–63.1)	25.4	98.1
>17.5	88.24 (63.5–98.2)	55.00 (44.7–65.0)	25.0	96.5
>34.0*	88.24 (63.5–98.2)	99.00 (94.5–99.8)	93.7	98.0
>34.7	82.35 (56.6–96.0)	99.00 (94.5–99.8)	93.3	97.1
>35.2	82.35 (56.6–96.0)	100.0 (96.3–100.0)	100.0	97.1
>98.4	0.00 (0.0–19.7)	100.0 (96.3–100.0)		85.5

Data in parentheses are 95% CI. *Cutoff value for positivity.

volunteers (n = 100) and fulminant type 1 diabetic patients (n = 17) (Fig. 2*F*). Table 2 shows criterion values and coordinates of the ROC curve. When the value was set as 34 (area under the ROC curve 0.92; significance level P = 0.0001), sensitivity, specificity, and positive predictive value were 88.24, 99.0, and 93.7%, respectively.

Prevalence of autoantibody against human AMY-2A in AIP patients. Using the ELISA system, we determined the prevalence of autoantibody against human AMY-2A in AIP patients and various pancreatic diseases (Fig. 3). All 15 IgGs from patients with AIP were positive for AMY-2A/299–512, whereas 1 of 100 IgGs from control subjects was positive for the antibody (P < 0.001, Fisher's exact test). All the IgGs from the patients with chronic alcoholic pancreatitis (n = 25) or with pancreas tumor (pancreatic cancer, n = 8; IPMT, n = 17) were negative for the antigen. Antibodies were detected in 9% (4/47) of patients with Hashimoto's thyroiditis, a representative organ-specific autoimmune disease (Fig. 3*A*).

Figure 3*B* shows the time course of the autoantibody titer from two AIP patients before and after prednisolone treatment. In patient A.O., IgG4 gradually increased and reached 5,540 mg/dl, but administration of prednisolone initiated a rapid decrease of IgG4 to 571 mg/dl. Before prednisolone treatment, the titer of the autoantibody against AMY remained high, and prednisolone treatment induced a rapid decrease of the titer of AMY-2A autoantibody to a normal level. The fall rate of the antibody titer seemed to be parallel to that of serum IgG4. In patient T.M., administration of prednisolone also rapidly decreased the titer of the autoantibody against AMY. The autoantibodies did not increase even at the drug maintenance dose in both cases.

Prevalence of autoantibody against human AMY-2A in patients with fulminant type 1 diabetes and acute-onset type 1 diabetes. We next studied the prevalence of autoantibody against human AMY-2A in various types of diabetic patients (fulminant type 1 diabetes, n = 17; acute-onset type 1 diabetes, n = 42; and type 2 diabetes, n = 67; Fig. 4). Interestingly, 88% of patients with fulminant type 1 diabetes were positive for the autoantibody, but 1% of control was positive for the antibody (P < 0.001, Fisher's exact test). The autoantibody was detected with low frequency in patients with acute-onset type 1 diabetes

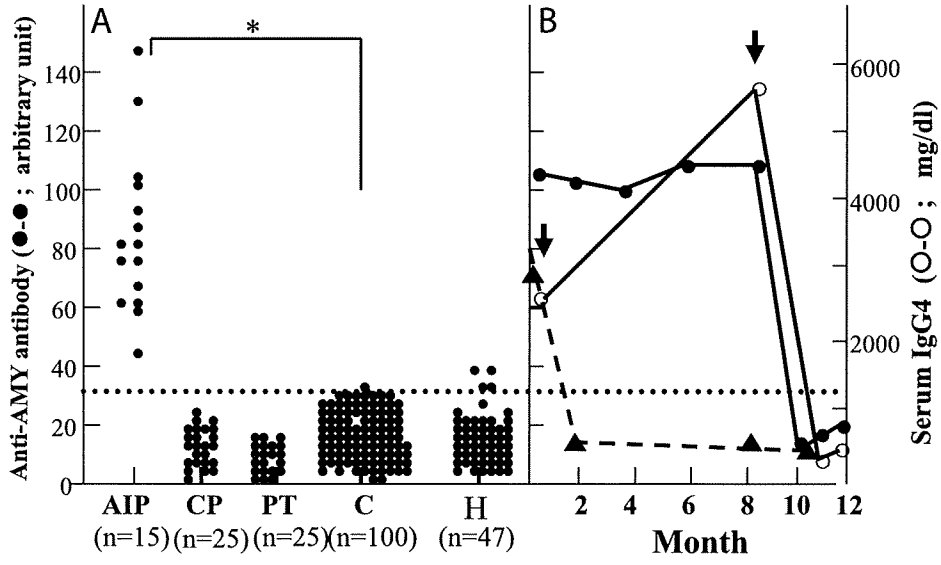

FIG. 3. Prevalence of autoantibody against human AMY-2A in patients with various pancreatic diseases. *A*: Prevalence of autoantibody against human AMY-2A in patients with AIP (n = 15), chronic alcoholic pancreatitis (CP, n = 25), pancreatic tumor (PT, n = 25), control subjects from healthy volunteers (C, n = 100), and Hashimoto's thyroiditis (H, n = 47) was examined by ELISA, as described in RESEARCH DESIGN AND METHODS. The data are the mean of triplicate values. The dotted line shows a cutoff value. Fisher's exact test was carried out between AIP and control groups. *P < 0.001. *B*: Time course of anti-AMY antibody and IgG4 of AIP patients. AIP patient (A.O.), whose IgG was used to screen λTriplEx2 human pancreas cDNA library, was treated with prednisolone (*arrow*). Before and after the treatment, anti-AMY antibody (●-●) and IgG4 (○-○) were measured. In the other AIP patient (T.M.), titer of the anti-AMY antibody (▲-▲) was also measured before and after prednisolone treatment (*arrow*).

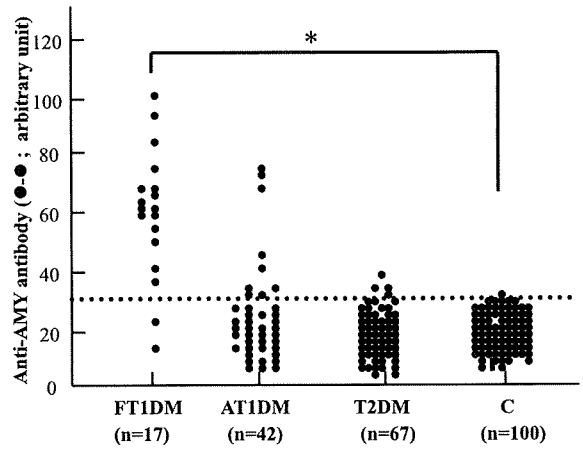

FIG. 4. Prevalence of autoantibody against human AMY-2A in patients with various types of diabetes. Prevalence of autoantibody against human AMY-2A (●) in patients with fulminant type 1 diabetes (FT1DM, n = 17), acute-onset type 1 diabetes (AT1DM, n = 42), type 2 diabetes (T2DM, n = 67), and control subjects from healthy volunteers (C, n = 100) was studied by ELISA, as described in RESEARCH DESIGN AND METHODS. The data are the mean of triplicate values. The dotted line shows a cutoff value. Fisher's exact test was carried out between fulminant type 1 diabetic and control groups. *P < 0.001.

(21%, 9 of 42) and patients with type 2 diabetes (6%, 4 of 67).

DISCUSSION

In 2002, Barera et al. (18) reported a case of an 11-year-old girl with celiac disease and hypothyroidism. Because of hyperamylasemia, she was suspected to have chronic pancreatitis, but no pancreatic damage was demonstrated. By using ELISA to detect autoantibodies to amylase, they found that she produced an autoantibody against porcine amylase and that this declined after the institution of a gluten-free diet. In the present study, we also detected an autoantibody against pancreas-specific AMY-2A in all of the AIP patients, but not in patients with chronic alcoholic pancreatitis and with pancreatic tumors.

The presence of autoantibodies against CAII, lactoferrin, and pancreatic secretory trypsin inhibitor (PSTI) has been reported (3,4,19). However, the distribution of these molecules is non–organ specific (20–22), and the prevalence of these autoantibodies against CAII, lactoferrin, and PSTI in AIP is rather low, ranging from 42–73% (3,4,19). Using 13 serum samples from our AIP patients, we carried out ELISA assays for autoantibodies against CAII and lactoferrin. As a result, 66% (10 of 15) were positive for CAII, and 53% (8 of 15) were positive for lactoferrin. Thus, an autoantibody against AMY-2A might be a more sensitive marker for AIP than that of CAII, lactoferrin, or PSTI.

Furthermore, the adoptive transfer of amylase-specific $CD4^+$ T-cells to rats was able to confer pancreatitis, whereas the transfer experiment with lactoferrin-specific or CAII-specific $CD4^+$ T-cells failed to induce experimental pancreatitis (23). Our findings of a high prevalence of autoantibody against AMY-2A in human AIP and the results from the adoptive transfer experiment of amylase-specific $CD4^+$ T-cells to rodents suggest that cellular and/or humoral autoimmunity against AMY-2A plays some role in the pathogenesis of AIP.

Approximately 80% of patients with chronic pancreatitis are alcoholic, the pathogenesis of which still remains unclear. However, it is well known that acute or chronic alcohol exposure suppresses all branches of the immune system (24), and none of our sera from patients with chronic alcoholic pancreatitis were positive for autoantibody against AMY-2A (Fig. 3). Therefore, an assay for autoantibody against AMY-2A is useful for distinguishing AIP from chronic alcoholic pancreatitis.

It is of particular interest that anti–AMY-2A autoantibody is detected in 88% of patients with fulminant type 1 diabetes. Fulminant type 1 diabetes is a recently proposed subtype of type 1B, nonimmune-mediated, or idiopathic type 1 diabetes (9,10). A nationwide survey revealed that fulminant diabetes accounted for ~20% of Japanese type 1 diabetes with ketosis or ketoacidosis and flu-like symptoms frequently observed at onset (25). Clinical characteristics of this subtype of type 1 diabetes are *1*) remarkably abrupt onset of disease; *2*) very short (<1 week) duration of diabetic symptoms; *3*) severe ketoacidosis at diagnosis; *4*) negative status of islet-related autoantibodies, such as GADAb and anti–IA-2 antibody; *5*) virtually no C-peptide secretion (10 μg/day in urine); and *6*) elevated serum pancreatic enzyme levels (26). These features and the absence of insulitis in patients' pancreases have led some to hypothesize that an autoimmune mechanism does not contribute to the development of fulminant type 1 diabetes, but rather that viral infection plays a central role in the pathogenesis of the disease (27). However, we previously demonstrated $CD4^+$ and $CD8^+$ T-cell infiltration to pancreatic exocrine cells and to the islet in an autopsy case deceased immediately after the onset of fulminant type 1 diabetes (28).

Imagawa and Hanafusa (27) also confirmed cellular infiltration of pancreatic islets in patients with fulminant type 1 diabetes. Shimada et al. (29) described a fulminant type 1 diabetic patient with a high serum level of CXCL10, a chemokine that induces migration of activated T-cells to local lesions and GAD-reactive $CD4^+$ cells in the periphery. These results, and the presence of an autoantibody against AMY-2A, suggest that the disease might be autoimmune-related, involving the exocrine and the endocrine pancreas (10,28).

Exocrine dysfunction and impaired glucose tolerance are common features for both AIP and fulminant type 1 diabetes. With regard to the HLA genotype, Kawa et al. (30) demonstrated that the DRB1*0405-DQB1*0401 haplotype is closely associated with AIP in the Japanese population, and Tanaka et al. (31) revealed that the DQA1*0303-DQB1*0401 haplotype is strongly associated with fulminant type 1 diabetes in a homologous manner. When we studied the frequency of this allele in our patients with AIP, 5 of 15 patients were heterozygous for the DRB1*0405-DQB1*0401 haplotype. Although further study with larger sample sizes will be needed, these two reports and our own analysis suggest the importance of the DQB1*0401 allele in both diseases. Furthermore, we are able to detect autoantibody against AMY-2A in both with nearly the same prevalence. Although further investigation is needed, the present results suggest that clinically and immunologically, AIP and fulminant type 1 diabetes are closely related to one another.

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ARTICLE

Differential association of HLA with three subtypes of type 1 diabetes: fulminant, slowly progressive and acute-onset

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Abstract

Aim/hypothesis We sought to clarify similarities and differences in the contribution of HLA to genetic susceptibility to three subtypes of type 1 diabetes: acute-onset, fulminant and slowly progressive.

Methods We genotyped 545 Japanese patients with type 1 diabetes (338 acute-onset, 80 fulminant, 127 slowly progressive) and 396 control participants at *HLA-DRB1*, *-DQB1*, *-A*, *-B* and *-C*, and at 101 candidate single nucleotide polymorphisms (SNPs) in an 8.5 Mb region of the extended HLA.

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Results *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302* and *DRB1*0901-DQB1*0303* were associated with acute-onset type 1 diabetes, with the *DRB1*0405-DQB1*0401/DRB1*0802-DQB1*0302* genotype achieving the highest odds ratio of 42.7. *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601* were negatively associated with acute-onset type 1 diabetes. A similar tendency was observed for slowly progressive type 1 diabetes. In contrast, only *DRB1*0405-DQB1*0401* was associated with fulminant type 1 diabetes, with the *DRB1*0405-DQB1*0401/DRB1*0405-DQB1*0401* genotype showing the highest odds ratio of 11.2. *DRB1*0802-DQB1*0302*, *DRB1*0405-DQB1*0401/DRB1*0802-DQB1*0302* and *DRB1*1501-DQB1*0602* were not associated with fulminant type 1 diabetes. The association of class I alleles and a panel of SNPs in an extended HLA region with fulminant type 1 diabetes was also different from that seen for the acute-onset and slowly progressive forms. The presence of both one and two susceptible haplotypes conferred susceptibility to slowly progressive type 1 diabetes, whereas the presence of two susceptible haplotypes was required to confer susceptibility to acute-onset and fulminant type 1 diabetes.
Conclusions/interpretation These data suggest that HLA associations with fulminant type 1 diabetes are qualitatively different from those with other subtypes of type 1 diabetes, whereas the HLA contribution to slowly progressive type 1 diabetes is qualitatively similar to, but quantitatively different from, that in acute-onset type 1 diabetes.

Keywords Fulminant type 1 diabetes · Genetics · HLA · Single nucleotide polymorphism · Slowly progressive type 1 diabetes · Susceptibility · Type 1 diabetes

Abbreviations

LADA	Latent autoimmune diabetes in adults
SNP	Single nucleotide polymorphism
UTR	Untranslated region

Introduction

Type 1 diabetes is a clinically and aetiologically heterogeneous disorder. In addition to typical acute-onset type 1 diabetes, at least two subtypes have been described: fulminant and slowly progressive. Fulminant type 1 diabetes, which is characterised by an extremely acute onset of diabetes and absence of islet-related autoantibodies [1], accounts for up to 20% of type 1 diabetes in Japan [2] and 7% in Korea [3]. Slowly progressive type 1 diabetes, in contrast, is characterised by positivity for islet-related autoantibodies, but a long non-insulin-dependent stage, lasting usually for years, with gradual loss of beta cells leading ultimately to an insulin-dependent stage [4]. Although all three subtypes share the same clinical characteristic of insulin dependence in the final stage, the time course of beta cell destruction is markedly different, which may well be based on differences in the aetiology, including genetic susceptibility, among the three subtypes of the disease [2, 4].

The contribution of HLA, in particular class II DR and DQ genes, to susceptibility to autoimmune type 1 diabetes has been well described [5]. The HLA alleles contributing to fulminant type 1 diabetes appear to be different from those in autoimmune type 1 diabetes. In an initial report [1], high frequencies of class II HLA alleles known to provide resistance to type 1 diabetes were described. Subsequent studies with serological typing of class II HLA in patients recruited through a nationwide survey showed a higher frequency of DR4-DQ4 as well as DR2-DQ1 haplotypes in fulminant type 1 diabetes than in autoimmune type 1 diabetes [6]. Slowly progressive type 1 diabetes was also reported to be associated with class II HLA [4].

Despite the contribution of HLA to each subtype of type 1 diabetes, no extensive studies comparing HLA alleles and genotypes in the three subtypes of type 1 diabetes have been performed, probably because the low frequency of type 1 diabetes in Japan makes it difficult to collect substantial numbers of samples for all three subtypes at any given institute. To overcome this, the Committee on Type 1 Diabetes of the Japan Diabetes Society has been performing nationwide studies and collecting samples from patients with fulminant as well as the typical acute-onset and slowly progressive subtypes of type 1 diabetes. Taking advantage of these samples, we studied the associations of a panel of single nucleotide polymorphism (SNP) markers in the HLA region as well as class I and class II alleles of HLA with all three subtypes of type 1 diabetes. Our aim was to clarify the similarities and differences in the contribution of HLA to genetic susceptibility to the three subtypes.

Methods

Participants We studied 545 Japanese patients with type 1 diabetes (338 typical acute-onset, 80 fulminant and 127 slowly progressive type 1 diabetes) and 396 healthy control participants. Patients with fulminant type 1 diabetes were recruited through the Japan Diabetes Society as described previously [6]. Samples from patients with acute-onset and slowly progressive type 1 diabetes were collected from the hospitals of the committee members. The patients were ketosis-prone and positive for at least one of the islet-related autoantibodies, i.e. GAD antibodies, islet cell antibodies, insulin autoantibodies and/or IA-2 antibodies.

The duration of hyperglycaemic symptoms before the start of insulin therapy was less than 3 months for typical acute-onset type 1 diabetes and more than 12 months for slowly progressive type 1 diabetes. This study was approved by the appropriate ethics committees and informed consent was obtained from all participants.

Genotyping of class II and class I HLA Class II *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. Class I A, B and C alleles were genotyped by PCR sequence-specific oligonucleotide method (Wakunaga Pharmaceutical, Hiroshima, Japan).

Genotyping of SNPs in HLA region A total of 101 SNPs in 76 candidate genes in an 8.5 Mb region of the HLA was selected. Selection of candidate genes and SNPs was based on the following criteria: candidate genes reported in the literature and identified on PubMed using the following keywords: type 1 diabetes, insulin-dependent diabetes mellitus, gene, human. In addition, genes related to immunological function and those expressed in the pancreas were also included. When multiple SNPs were identified in the same gene, SNPs were prioritised as follows: (1) SNPs reported to have a significant association with the disease; (2) SNPs in coding sequences; (3) SNPs in promoter, 5′ and 3′ untranslated regions; and (4) SNPs in introns. Within the same category, priority was given to SNPs uploaded in JSNP (http://snp.ims.u-tokyo.ac.jp/index.html, accessed 4 March 2009) with minor allele frequencies >0.05 in Japanese.

Genotyping was performed by the PCR fluorescence correlation spectroscopy method at the SNP Typing Center in the Department of Human Genetics, Tokyo University, as reported previously [7]. Initially, 16 samples commonly used at the Typing Center for pilot screening for polymorphisms were typed, with only SNPs confirmed to show polymorphism in at least one sample being used for further typing. Nine SNPs not polymorphic in the initial screening and 24 SNPs that showed either non-specific amplification due to homologous pseudogenes or low calling rates were omitted. The remaining 68 SNPs were used for typing of all samples.

Statistical analysis Statistical analysis was performed with StatView 5.0 (SAS Institute, Cary, NC, USA). The significance of differences in the distribution of alleles was determined by the χ^2 test or Fisher's exact probability test. p values were corrected for the number of different alleles tested (denoted as p_c). Statistical significance was defined as $p<0.05$.

Results

Class II alleles and haplotypes Association of class II HLA alleles and haplotypes with all three subtypes of type 1 diabetes was observed (Table 1, Electronic supplementary material [ESM] Tables 1, 2 and 3). However, a marked difference in the alleles and haplotypes associated with the disease was observed among the three subtypes. As reported previously [8–11], the *DRB1*0405* and **0901* alleles (ESM Table 1), *DQB1*0401* and **0303* alleles (ESM Table 2), and *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302* and *DRB1*0901-DQB1*0303* haplotypes (Table 1) were associated with acute-onset type 1 diabetes. The *DRB1*1501* and **1502* alleles (ESM Table 1), *DQB1*0601* and *DQB1*0602* alleles (ESM Table 2), and *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601* haplotypes (Table 1) were negatively associated with acute-

Table 1 *DRB1-DQB1* haplotypes associated positively (susceptible) or negatively (protective) with disease in patients with acute-onset, fulminant and slowly progressive type 1 diabetes, and in control participants

DRB1-DQB1	Control (n=792)		Acute (n=676)		Fulminant (n=160)		SP (n=254)		A vs C		F vs C		SP vs C	
	n	%	n	%	n	%	n	%	p_c value	OR	p_c value	OR	p_c value	OR
Susceptible														
**0405-*0401*	96	12.1	205	30.3	51	31.9	65	25.6	2.6×10^{-16}	3.2	1.2×10^{-7}	3.4	4.5×10^{-6}	2.5
**0802-*0302*	14	1.8	48	7.1	3	1.9	13	5.1	1.6×10^{-5}	4.3	NS	1.1	NS	3.0
**0901-*0303*	117	14.8	209	30.9	38	23.8	62	24.4	3.8×10^{-12}	2.6	NS	1.8	7.8×10^{-3}	1.9
Protective														
**1501-*0602*	59	7.4	6	0.9	11	6.9	7	2.8	4.9×10^{-8}	0.11	NS	0.92	NS	0.35
**1502-*0601*	80	10.1	21	3.1	8	5.0	4	1.6	4.6×10^{-6}	0.29	NS	0.47	2.7×10^{-4}	0.14

For complete list of haplotypes including those not associated with the disease, see ESM Table 3

A, acute-onset; C, control participants; F, fulminant; p_c, p values corrected for number of different haplotypes tested; SP, slowly progressive

onset type 1 diabetes. The alleles and haplotypes associated with slowly progressive type 1 diabetes were similar to those associated with acute-onset type 1 diabetes (Table 1, ESM Tables 1, 2 and 3). In contrast, the alleles and haplotypes associated with fulminant type 1 diabetes were different from those associated with other subtypes. The strong negative association of the *DRB1*1501* and **1502* alleles, the *DQB1*0602* alleles and the *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601* haplotypes with acute-onset type 1 diabetes was not observed in fulminant type 1 diabetes. In fact, the frequency of a strongly protective haplotype, *DRB1*1501-DQB1*0602*, was significantly higher in fulminant type 1 diabetes than in acute-onset type 1 diabetes (6.9% vs 0.9%, $p_c=2.8\times10^{-5}$) and was similar to that in control participants (7.4%). The positive association of the *DRB1*0802-DQB1*0302* haplotype observed in acute-onset type 1 diabetes (OR 4.3, $p_c=1.6\times10^{-5}$) was not observed in fulminant type 1 diabetes (OR 1.1, NS) (Table 1).

Class II genotypes Homozygosity for *DRB1*0405-DQB1*0401* was associated with all three subtypes of type 1 diabetes. The frequency, however, was much higher in fulminant type 1 diabetes (12.5%, OR 11.2) than in the acute-onset (8.3%, OR 7.1) and slowly progressive (7.1%, OR 6.0) subtypes, although the difference between the groups was not statistically significant (Table 2). In contrast, the frequency of *DRB1*0901-DQB1*0303* homozygotes was higher in acute-onset (15.7%) than in fulminant (7.5%) and slowly progressive (7.9%) type 1 diabetes (Table 2). The *DR4/8* (*DRB1*0405-DQB1*0401/DRB1*0802-DQB1*0302*) genotype was significantly associated with acute-onset (OR 42.7, $p_c=5.6\times10^{-10}$) and slowly progressive (OR 16.2, $p_c=0.03$) type 1 diabetes, but not with fulminant type 1 diabetes (Table 1); the frequency in fulminant type 1 diabetes was lower than in acute-onset type 1 diabetes (9.8% vs 1.3%, $p=0.01$).

On the basis of their association with acute-onset type 1 diabetes, haplotypes were classified as susceptible (S) (haplotypes *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302* and *DRB1*0901-DQB1*0303*), protective (P) (haplotypes *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601*) or neutral (N) (haplotypes other than susceptible and protective haplotypes). Within this classification, the presence of two susceptible haplotypes (S/S) was associated with acute-onset (OR 10.0, $p_c=2.6\times10^{-35}$) and fulminant type 1 diabetes (OR 5.7, $p_c=4.7\times10^{-8}$), but the presence of one susceptible haplotype (S/N, S/P) had no effect on susceptibility to the disease (Table 3). In contrast, the presence of both two (S/S) and of one (S/N) susceptible haplotype was associated with slowly progressive type 1 diabetes (OR 4.1, $p_c=8.5\times10^{-8}$; OR 2.4, $p_c=1.1\times10^{-4}$, respectively) (Table 3).

Protective haplotypes provided strong protection against acute-onset (OR 0.17, $p_c=2.0\times10^{-17}$) and slowly progressive type 1 diabetes (OR 0.19, $p_c=3.4\times10^{-7}$), but no such effect was observed for fulminant type 1 diabetes (OR 0.58, NS) (Table 3). Thus, the susceptibility and protection provided by HLA haplotypes differed among the three subtypes of type 1 diabetes. In acute-onset type 1 diabetes S/S provided susceptibility and P provided protection, while S/S provided susceptibility but with no protective haplotypes in fulminant type 1 diabetes; in the slowly progressive subtype, both S/S and S/N provided susceptibility and P provided protection.

Class I HLA alleles and genotypes As seen in ESM Tables 4, 5, and 6, the frequency of *B*5401* was

Table 2 *DRB1-DQB1* genotypes in patients with acute-onset, fulminant and slowly progressive type 1 diabetes, and in control participants

DRB1-DQB1	Control (n=396)		Acute (n=338)		Fulminant (n=80)		SP (n=127)		A vs C		F vs C		SP vs C	
	n	%	n	%	n	%	n	%	p_c value	OR	p_c value	OR	p_c value	OR
DR4/4	5	1.3	28	8.3	10	12.5	9	7.1	4.3×10^{-5}	7.1	1.4×10^{-6}	11.2	0.014	6.0
DR9/9	12	3.0	53	15.7	6	7.5	10	7.9	9.8×10^{-9}	6.0	NS	2.6	NS	2.7
DR4/9	15	3.8	46	13.6	11	13.8	12	9.4	1.4×10^{-5}	4.0	0.013	4.1	NS	2.7
DR4/8	1	0.3	33	9.8	1	1.3	5	3.9	5.6×10^{-10}	42.7	NS	5.0	0.034	16.2
DR8/9	3	0.8	9	2.7	1	1.3	1	0.8	NS	3.6	NS	1.7	NS	1.0
DR4/X	70	17.7	70	20.7	19	23.8	30	23.6	NS	1.2	NS	1.5	NS	1.4
DR9/X	75	18.9	48	14.2	14	17.5	29	22.8	NS	0.71	NS	0.91	NS	1.3
DR8/X	10	2.5	6	1.8	1	1.3	7	5.5	NS	0.70	NS	0.49	NS	2.3
DRX/X	205	51.8	45	13.3	17	21.3	24	18.9	1.9×10^{-28}	0.14	5.4×10^{-6}	0.25	2.2×10^{-10}	0.2

DR4: *DRB1*0405-DQB1*0401* haplotype; DR8: *DRB1*0802-DQB1*0302* haplotype; DR9: *DRB1*0901-DQB1*0303* haplotype; X: haplotypes other than *DR4*, *DR8* and *DR9*

A, acute-onset; C, control participants; F, fulminant; p_c, p values corrected for number of different haplotypes tested; SP, slowly progressive

Table 3 *DRB1-DQB1* genotypes in patients with acute-onset, fulminant and slowly progressive type 1 diabetes, and in control participants

DRB1-DQB1	Control (n=396)		Acute (n=338)		Fulminant (n=80)		SP (n=127)		A vs C		F vs C		SP vs C	
	n	%	*n*	%	*n*	%	*n*	%	p_c value	OR	p_c value	OR	p_c value	OR
S/S	36	9.1	169	50.0	29	36.3	37	29.1	2.6×10^{-35}	10.0	4.7×10^{-8}	5.7	8.5×10^{-8}	4.1
S/N	109	27.5	109	32.2	27	33.8	61	48.0	NS	1.3	NS	1.3	1.1×10^{-4}	2.4
S/P	46	11.6	15	4.4	7	8.8	5	3.9	2.7×10^{-3}	0.35	NS	0.73	NS	0.31
N/N	119	30.1	34	10.1	6	7.5	18	14.2	8.3×10^{-11}	0.26	1.7×10^{-4}	0.19	2.4×10^{-3}	0.38
N/P	78	19.7	10	3.0	10	12.5	6	4.7	1.2×10^{-12}	0.12	NS	0.58	3.8×10^{-4}	0.20
P/P	8	2.0	1	0.3	1	1.3	0	0.0	NS	0.14	NS	0.61	NS	–
Non-S/non-S	205	51.8	45	13.3	17	21.3	24	18.9	1.3×10^{-28}	0.14	3.6×10^{-6}	0.25	1.5×10^{-10}	0.22
P/X	132	33.3	26	7.7	18	22.5	11	8.7	2.0×10^{-17}	0.17	NS	0.58	3.4×10^{-7}	0.19

A, acute-onset; C, control participants; F, fulminant; P, protective haplotypes against acute-onset type 1 diabetes, *DRB1*1501-DQB1*0602* and *DRB1*1502-DQB1*0601*; p_c, p values corrected by multiplying by number of genotypes (×6); S, susceptible haplotypes for acute-onset type 1 diabetes, *DRB1*0405-DQB1*0401*, *DRB1*0802-DQB1*0302* and *DRB1*0901-DQB1*0303*; SP, slowly progressive; X, any haplotype (e.g. P/X= P/S+P/N+P/P)

significantly higher in acute-onset (OR 2.1, $p_c=2.9\times10^{-3}$) and slowly progressive diabetes (OR 2.6, $p_c=1.6\times10^{-2}$) than in control participants, but this was not the case for fulminant type 1 diabetes (OR 1.2, NS) (ESM Table 5). In contrast, the frequency of *B*4002* was significantly higher in fulminant (OR 2.9, $p_c=0.017$), but not in acute-onset (OR 1.4, NS) and slowly progressive (OR 0.94, NS) type 1 diabetes, as compared with that in control participants (ESM Table 5). The frequency of *C*0803* was significantly higher in fulminant (OR 9.6, $p_c=0.022$), but not in acute-onset and slowly progressive type 1 diabetes, than in control participants (ESM Table 6); it was also significantly higher in fulminant than in acute-onset type 1 diabetes ($p_c=0.03$). The frequency of *C*0801* was significantly higher in acute-onset, but not in fulminant and slowly progressive type 1 diabetes, than in control participants (ESM Table 6). The frequencies of *B*5201* (ESM Table 5) and *C*1202* (ESM Table 6) were significantly lower in acute-onset (OR 0.35, $p_c=5.1\times10^{-3}$; OR 0.37, $p_c=4.4\times10^{-3}$, respectively), but not in fulminant and slowly progressive type 1 diabetes, than in control participants.

Since most class I alleles showing association with type 1 diabetes were reported to be on haplotypes containing disease-susceptible class II HLA in the Japanese population [12], the participants were stratified by *DRB1-DQB1* to investigate whether or not these associations were secondary to linkage disequilibrium with *DRB1-DQB1* haplotypes. Class I alleles associated with acute-onset type 1 diabetes were in strong linkage disequilibrium with class II *DRB1-DQB1* haplotypes conferring susceptibility or resistance to acute-onset type 1 diabetes. The frequency of *B*5401* was much higher in patients with *DRB1*0405-DQB1*0401* than in those without (46.5% vs 15.9%, $p=6.8\times10^{-7}$), and the association of *B*5401* with the disease was observed only in patients with *DRB1*0405-DQB1*0401* (acute-onset: OR 4.0, $p=3.7\times10^{-8}$), but not in those without it (OR 0.86, NS) (ESM Table 7). Similarly, *B*4006* and *C*0801* were in linkage disequilibrium with *DRB1*0901-DQB1*0303* (37.3% vs 12.0%, $p=8.7\times10^{-6}$ and 40.2% vs 17.4%, $p=1.1\times10^{-4}$ in patients with and without *DRB1*0901-DQB1*0303*, respectively); their association with the disease was observed in patients with, but not in patients without *DRB1*0901-DQB1*0303* (ESM Table 7). *B*5201* and *C*1202*, which were negatively associated with the disease, were in linkage disequilibrium with *DRB1*1502-DQB1*0601* (40.0% vs 5.2%, $p=2.3\times10^{-4}$ and 43.7% vs 5.1%, $p=3.6\times10^{-5}$ in patients with and without *DRB1*1502-DQB1*0601*, respectively). In contrast, the association of *B*4002* with fulminant type 1 diabetes was observed regardless of the presence or absence of *DRB1*0405-DQB1*0401* and *DRB1*0901-DQB1*0303* (ESM Table 7).

Association with SNPs in HLA region As seen in Table 4 and ESM Fig. 1, a SNP located in class II *DQB1* (rs1049107) was associated with all three subtypes, confirming that association with class II HLA is observed in all three subtypes of type 1 diabetes. A SNP located in *TNF* (rs1800610) was also associated with all three subtypes.

In addition to the peaks observed in all three subtypes, several peaks limited to one or two subtypes were observed. Several SNPs showed an association with fulminant, but not with acute-onset and slowly progressive type 1 diabetes. Among these were: (1) rs2071800, located in the coding region of *DQA2*; (2) rs2071552 and rs3763364 located in the 5′ untranslated region (UTR) and promoter region respectively of *TAP2*; and (3) rs2294689 located in the coding region of *TTRAP*.

Table 4 Association of SNPs typed in the HLA region with acute, fulminant and slowly progressive type 1 diabetes

SNP ID	dbSNP ID (rs no.)	Gene symbol	Location	OR (95% CI)			*p* value		
				Acute	Fulminant	SP	Acute	Fulminant	SP
001	2294689	*TTRAP*	CDS	1.28 (0.99–1.65)	1.96 (1.31–2.92)	1.45 (1.04–2.02)	NS	0.0009	0.03
002	2275906	*SLC17A4*	CDS	1.00 (0.59–1.71)	0.65 (0.25–1.73)	0.79 (0.38–1.67)	NS	NS	NS
003	1572982	*HFE*	Intron	0.99 (0.68–1.43)	1.28 (0.75–2.20)	1.08 (0.67–1.73)	NS	NS	NS
004	3736781	*BTN1A1*	CDS	1.12 (0.85–1.48)	0.66 (0.41–1.05)	0.98 (0.68–1.40)	NS	NS	NS
005	3734576	*PRSS16*	3′UTR	0.76 (0.42–1.38)	0.75 (0.28–2.01)	1.00 (0.48–2.07)	NS	NS	NS
006	2294481	*D6S2223*	Intron	1.12 (0.87–1.44)	1.05 (0.71–1.56)	1.23 (0.88–1.71)	NS	NS	NS
007	1480646	*ZNF192*	Promoter	1.03 (0.73–1.45)	1.23 (0.74–2.05)	0.91 (0.58–1.45)	NS	NS	NS
008	2269553	*TRIM27*	Intron	1.31 (1.02–1.69)	1.13 (0.76–1.68)	1.59 (1.15–2.22)	0.03	NS	0.006
009	29230	*GABBR1*	CDS	0.74 (0.54–1.02)	0.72 (0.43–1.19)	0.99 (0.67–1.46)	NS	NS	NS
010	2252711	*MOG*	Intron	1.03 (0.76–1.40)	0.77 (0.47–1.28)	1.42 (0.98–2.08)	NS	NS	NS
011	1736922	*HLA-F*	Intron	1.46 (1.13–1.88)	1.15 (0.77–1.72)	0.90 (0.64–1.27)	0.004	NS	NS
012	378971	*HCG9*	CDS	0.69 (0.53–0.90)	0.94 (0.63–1.40)	0.93 (0.66–1.30)	0.005	NS	NS
013	2074479	*RNF39*	CDS	0.96 (0.71–1.29)	1.59 (1.03–2.45)	1.01 (0.69–1.47)	NS	0.04	NS
014	2074474	*TRIM39*	CDS	1.16 (0.89–1.49)	1.05 (0.70–1.57)	1.44 (1.03–2.00)	NS	NS	0.03
015	3757388	*IRF5*	Promoter	0.12 (0.01–2.40)	1.22 (0.13–11.80)	0.72 (0.07–6.99)	NS	NS	NS
016	1265054	*C6orf15*	CDS	0.69 (0.53–0.90)	0.79 (0.53–1.19)	0.74 (0.52–1.03)	0.005	NS	NS
017	2073721	*TCF19*	CDS	1.01 (0.78–1.31)	1.04 (0.69–1.56)	1.29 (0.92–1.80)	NS	NS	NS
018	2523946	*HCG9*	Promoter	0.79 (0.60–1.03)	0.75 (0.49–1.14)	1.08 (0.77–1.52)	NS	NS	NS
019	1049853	*HLA-C*	3′UTR	1.90 (1.28–2.83)	2.10 (1.20–3.68)	1.61 (0.97–2.67)	0.001	0.008	NS
020	3819300[a]	*HLA-B*	CDS	0.56 (0.38–0.82)	0.64 (0.35–1.17)	0.95 (0.61–1.50)	0.003	NS	NS
021	709052	*HLA-B*	CDS	0.66 (0.49–0.90)	0.47 (0.27–0.82)	0.86 (0.58–1.27)	0.009	0.007	NS
022	1050747	*HLA-B*	CDS	0.73 (0.51–1.04)	1.10 (0.65–1.86)	0.59 (0.35–0.97)	NS	NS	0.04
023	2534674	*MICB*	Promoter	1.38 (1.06–1.79)	1.05 (0.70–1.58)	1.42 (1.02–1.98)	0.02	NS	0.04
024	2239527	*BAT1*	5′UTR	0.77 (0.59–1.01)	0.78 (0.51–1.18)	0.85 (0.60–1.20)	NS	NS	NS
025	2230365	*NFKBIL1*	CDS	1.01 (0.76–1.35)	1.31 (0.85–2.03)	0.88 (0.60–1.29)	NS	NS	NS
026	2239704	*LTA*	5′UTR	1.02 (0.79–1.31)	0.82 (0.55–1.23)	0.95 (0.68–1.32)	NS	NS	NS
027	1800610	*TNF*	Intron	2.87 (2.16–3.82)	1.95 (1.27–2.99)	2.17 (1.51–3.11)	1.0×10^{-13}*	0.002	2.0×10^{5}*
028	2256974	*LST1*	Intron	0.80 (0.62–1.05)	0.72 (0.47–1.11)	0.87 (0.62–1.24)	NS	NS	NS
029	2736176	*BAT2*	Promoter	1.12 (0.87–1.44)	1.17 (0.79–1.74)	1.08 (0.78–1.50)	NS	NS	NS
030	1046089	*BAT2*	CDS	1.31 (1.00–1.71)	1.38 (0.92–2.08)	1.05 (0.74–1.50)	0.047	NS	NS
031	2242656	*BAT3*	Intron	1.04 (0.69–1.56)	1.31 (0.73–2.37)	1.53 (0.95–2.48)	NS	NS	NS
032	7992	*BAT4*	CDS	1.36 (1.05–1.76)	1.34 (0.89–2.00)	1.29 (0.92–1.80)	0.02	NS	NS
033	805282	*BAT5*	Intron	1.32 (1.02–1.71)	1.28 (0.86–1.91)	1.32 (0.95–1.85)	0.04	NS	NS
034	2075800	*HSPA1L*	CDS	0.96 (0.74–1.24)	0.91 (0.61–1.35)	0.92 (0.66–1.29)	NS	NS	NS
035	7887	*EHMT2*	CDS	0.69 (0.52–0.91)	0.56 (0.35–0.89)	0.93 (0.65–1.32)	0.01	0.01	NS
036	2072634	*CFB*	CDS	0.55 (0.33–0.90)	0.50 (0.21–1.22)	0.61 (0.31–1.18)	0.02	NS	NS
037	3749966	*C6orf10*	CDS	0.81 (0.59–1.11)	0.45 (0.25–0.83)	0.95 (0.64–1.42)	NS	0.009	NS
038	2076530	*BTNL2*	CDS	1.18 (0.92–1.53)	0.97 (0.65–1.46)	1.27 (0.91–1.77)	NS	NS	NS
039	14004	*HLA-DRA*	5′UTR	1.36 (1.04–1.77)	1.12 (0.74–1.69)	1.28 (0.91–1.80)	0.02	NS	NS
040	1049107	*HLA-DQB1*	CDS	0.20 (0.11–0.35)	0.19 (0.07–0.54)	0.27 (0.13–0.56)	6.7×10^{-10}*	5.6×10^{-4}*	1.7×10^{-4}*
041	2071800	*HLA-DQA2*	CDS	1.85 (1.01–3.41)	4.02 (1.95–8.29)	1.23 (0.53–2.82)	0.045	5.9×10^{-5}*	NS
042	3213484	*HLA-DQB2*	CDS	0.83 (0.62–1.10)	0.58 (0.35–0.95)	0.91 (0.63–1.32)	NS	0.03	NS
043	1049110	*HLA-DQB2*	CDS	0.85 (0.64–1.14)	0.56 (0.34–0.92)	0.91 (0.63–1.32)	NS	0.02	NS
044	2071554	*HLA-DOB*	CDS	0.34 (0.22–0.52)	0.66 (0.37–1.21)	0.28 (0.15–0.54)	4.2×10^{-6}*	NS	6.4×10^{-5}*
045	241441	*TAP2*	CDS	0.88 (0.68–1.14)	0.80 (0.53–1.20)	1.24 (0.88–1.74)	NS	NS	NS
046	2071552	*TAP2*	5′UTR	1.25 (0.97–1.61)	1.80 (1.21–2.67)	1.30 (0.93–1.81)	NS	0.004	NS
047	3763364	*TAP2*	Promoter	1.31 (1.00–1.71)	2.19 (1.46–3.28)	1.21 (0.86–1.71)	0.048	1.2×10^{-4}*	NS

Table 4 (continued)

SNP ID	dbSNP ID (rs no.)	Gene symbol	Location	OR (95% CI)			*p* value		
				Acute	Fulminant	SP	Acute	Fulminant	SP
048	2071543	*PSMB8*	CDS	0.70 (0.48–1.03)	1.32 (0.78–2.23)	0.64 (0.38–1.08)	NS	NS	NS
049	2071463	*PSMB8*	5′UTR	1.10 (0.85–1.43)	0.85 (0.56–1.29)	1.03 (0.73–1.45)	NS	NS	NS
050	1800453 [b]	*TAP1*	CDS	0.94 (0.62–1.41)	0.48 (0.21–1.08)	1.19 (0.72–1.97)	NS	NS	NS
051	2071536	*TAP1*	CDS	0.31 (0.18–0.56)	0.64 (0.29–1.39)	0.37 (0.17–0.80)	3.4×10^{-5}*	NS	0.009
052	17587	*PSMB9*	CDS	1.37 (1.02–1.84)	1.68 (1.08–2.60)	1.42 (0.97–2.07)	0.04	0.02	NS
053	1042337	*HLA-DMB*	CDS	1.05 (0.80–1.38)	1.04 (0.68–1.58)	1.08 (0.76–1.52)	NS	NS	NS
054	150359	*HLA-DMA*	Promoter	1.32 (1.02–1.70)	1.02 (0.68–1.51)	1.46 (1.05–2.03)	0.03	NS	0.03
055	516535	*BRD2*	CDS	1.30 (1.01–1.68)	1.01 (0.68–1.49)	1.44 (1.04–2.00)	0.04	NS	0.03
056	375256	*HLA-DOA*	CDS	1.16 (0.88–1.55)	1.02 (0.65–1.60)	0.88 (0.59–1.29)	NS	NS	NS
057	3097671	*HLA-DPB1*	Intron	0.56 (0.38–0.83)	0.68 (0.37–1.25)	0.48 (0.28–0.84)	0.003	NS	0.009
058	1799908	*COL11A2*	CDS	0.87 (0.64–1.17)	0.61 (0.36–1.02)	0.87 (0.59–1.29)	NS	NS	NS
059	2072915	*RXRB*	3′UTR	0.88 (0.65–1.20)	0.67 (0.40–1.12)	0.87 (0.58–1.30)	NS	NS	NS
060	383711	*HSD17B8*	Intron	0.54 (0.42–0.70)	0.64 (0.43–0.96)	0.59 (0.42–0.83)	2.7×10^{-6}*	0.03	0.002
061	213208	*RING1*	Intron	0.68 (0.52–0.89)	0.81 (0.53–1.24)	0.64 (0.44–0.92)	0.005	NS	0.02
062	213199	*VPS52*	CDS	0.67 (0.51–0.89)	1.02 (0.67–1.55)	0.54 (0.36–0.80)	0.005	NS	0.002
063	466384[c]	*WDR46*	CDS	0.94 (0.54–1.62)	0.40 (0.12–1.36)	0.65 (0.29–1.47)	NS	NS	NS
064	456261	*PFDN6*	Intron	0.66 (0.50–0.88)	0.95 (0.62–1.46)	0.57 (0.39–0.85)	0.004	NS	0.005
065	1059288	*TAPBP*	3′UTR	0.66 (0.50–0.87)	0.97 (0.64–1.47)	0.57 (0.39–0.83)	0.003	NS	0.003
066	2071888	*TAPBP*	CDS	0.65 (0.49–0.85)	0.93 (0.61–1.41)	0.57 (0.40–0.83)	0.002	NS	0.003
067	2073525	*DAXX*	Promoter	0.65 (0.49–0.85)	0.91 (0.60–1.37)	0.59 (0.41–0.86)	0.002	NS	0.005
068	2274730	*ZBTB9*	5′UTR	0.60 (0.45–0.81)	0.94 (0.60–1.48)	0.72 (0.50–1.06)	6.5×10^{-4}*	NS	NS

[a] Now merged into rs2308655; [b] now merged into rs1135216; [c] now merged into rs14398

*p values that remained significant after correction for number of SNPs genotyped (×68)

In contrast, SNPs located in the coding regions of *DOB* (rs2071554) and *TAP1* (rs2071536) were associated with acute-onset and slowly progressive, but not with fulminant type 1 diabetes. Similarly, an association was suggested between several SNPs located in the most centromeric region, e.g. rs3097671 in *DPB1* and rs383711 in *HSD17B8*, and both acute-onset and slowly progressive, but not fulminant type 1 diabetes. SNPs located in the region centromeric to *DPB1* (rs213208, rs213199, rs45261, rs1059288, rs2071888 and rs2073525) also showed a tendency for association with acute-onset and slowly progressive, but not fulminant type 1 diabetes.

To investigate whether or not these associations were secondary to linkage disequilibrium with susceptible and protective *DRB1-DQB1* haplotypes, the participants were stratified by *DRB1-DQB1*. A minor T allele of *DQA2*, which was strongly associated with fulminant type 1 diabetes (OR 4.02, $p=6\times10^{-5}$), was in strong linkage disequilibrium with *DRB1*0405-DQB1*0401* in control participants, with 19.1% frequency in participants with, as compared with 2.8% frequency in participants without *DRB1*0405-DQB1*0401* ($p=4.0\times10^{-5}$). The association of *DQA2*T* with the disease was observed only in patients with *DRB1*0405-DQB1*0401* (OR 10.3, $p=1.6\times10^{-8}$), but not in patients without *DRB1*0405-DQB1*0401* (OR 0.63, NS).

In contrast, SNPs whose minor alleles showed a negative association with acute-onset and slowly progressive type 1 diabetes were in linkage disequilibrium with protective *DRB1-DQB1* haplotypes, i.e. either *DRB1*1501* (rs2071536 in *TAP1*, $p=1.8\times10^{-14}$) or *DRB1*1502* (rs383711: $p=5.6\times10^{-5}$, rs45261: $p=2.4\times10^{-6}$, rs1059288: $p=4.0\times10^{-6}$, rs2071888: $p=3.5\times10^{-6}$, rs2073525: $p=7.8\times10^{-6}$) or both (rs2071554 in *DOB*, $p=1.8\times10^{-10}$ for *DRB1*1501* and $p=2.3\times10^{-21}$ for *DRB1*1502*).

Discussion

The present study demonstrates that class II HLA is associated with all three subtypes of type 1 diabetes, but the alleles, haplotypes and genotypes associated with the disease are markedly different among the three subtypes. Basically, the alleles and haplotypes associated with acute-

onset and slowly progressive type 1 diabetes were similar, whereas those associated with fulminant type 1 diabetes were mostly different from those in the other two subtypes of type 1 diabetes, as shown by the lack, in fulminant type 1 diabetes, of (1) protection conferred by *DRB1*1501-DQB1*0602*, a highly protective haplotype against acute-onset type 1 diabetes, and (2) susceptibility conferred by the *DRB1*0802-DQB1*0302* and *DR4/8* genotype, which confers strong susceptibility to acute-onset type 1 diabetes.

*DRB1*0405-DQB1*0401* was associated with fulminant as well as acute-onset and slowly progressive type 1 diabetes, but the magnitude of the effect differed, particularly in the homozygous form, playing a key role in fulminant type 1 diabetes. The fact that neither *DRB1* 1501-DQB1*0602* nor *DQB1*0302* affected susceptibility and only Asian-specific *DRB1*0405-DQB1*0401* conferred susceptibility in fulminant type 1 diabetes may explain the marked difference in incidence of fulminant type 1 diabetes among different ethnic groups. Fulminant type 1 diabetes has been reported in Asian populations, comprising up to 20% of adult-onset type 1 diabetes in Japan [2] and 7% of Korean type 1 diabetes [3]; in these populations *DRB1* 0405-DQB1*0401* is a common haplotype. In contrast, fulminant type 1 diabetes appears to be extremely rare in white populations [2, 13], in whom the *DRB1*0405-DQB1*0401* haplotype is also very rare. This may also reflect the difference in aetiology between fulminant and other subtypes of type 1 diabetes, i.e. autoimmune aetiology in acute-onset and slowly progressive type 1 diabetes, and idiopathic aetiology in fulminant type 1 diabetes [1, 2]. Several lines of evidence suggest viral infection in genetically susceptible individuals as a cause of fulminant type 1 diabetes [2, 14–16]. *DRB1*0405-DQB1*0401* was reported to be associated with immunological responses against certain viruses [17].

Slowly progressive type 1 diabetes is similar, but not identical to latent autoimmune diabetes in adults (LADA) in white populations [18] and is defined by autoimmune aetiology as reflected by positivity for islet-related autoantibodies, but slower progression to an insulin-dependent stage than in acute-onset type 1 diabetes [4]. LADA is defined by positivity for islet-related autoantibodies as in the case of slowly progressive type 1 diabetes, but progression to an insulin-dependent stage is not a necessary part of its definition [18]. Slowly progressive type 1 diabetes is more likely to be a mild form of acute-onset type 1 diabetes [4]. Consistent with this, the class II alleles and haplotypes associated with slowly progressive type 1 diabetes in the present study were similar to those in acute-onset type 1 diabetes. The similarity of HLA alleles and haplotypes between acute-onset and slowly progressive type 1 diabetes suggests that the phenotypic difference between these two subtypes of type 1 diabetes may not be due to differences in alleles and haplotypes of class II HLA. One possibility is a difference in presence of susceptible haplotypes between slowly progressive and acute-onset type 1 diabetes. As shown in Table 3, the presence of both two (S/S) and of one (S/N) susceptible haplotypes conferred susceptibility to slowly progressive type 1 diabetes, whereas for acute-onset type 1 diabetes only the former (S/S), but not the latter (S/N) conferred susceptibility, suggesting that differences in numbers of susceptible haplotypes affect the speed of disease progression, leading to the difference between acute-onset and slowly progressive forms of type 1 diabetes.

In addition to class II HLA, several loci, including class I HLA, showed some evidence of association with type 1 diabetes. Most alleles of class I HLA that were associated with acute-onset and slowly progressive type 1 diabetes (ESM Tables 4, 5, and 6) appeared to be secondary to linkage disequilibrium between these alleles and disease-related class II alleles, e.g. *B*5401* with *DRB1*0405-DQB1* 0401*, *B*4006* and *C*0801* with *DRB1*0901-DQB1*0303*, and *B*5201* and *C*1202* with *DRB1*01502-DQB1*0601*. However, the class I alleles associated with fulminant type 1 diabetes were different from those associated with acute-onset type 1 diabetes in that *B*5401*, which was increased in acute-onset and slowly progressive type 1 diabetes, was not increased and instead *B*4002* was increased. *B*4002* was reported to be on haplotypes containing *DRB1*0405-DQB1* 0401* and *DRB1*0901-DQB1*0303* in the Japanese population, although the haplotype frequencies are very low [12]. However, association of *B*4002* with fulminant type 1 diabetes cannot be explained by linkage disequilibrium with these class II haplotypes, because the association was observed regardless of the presence or absence of the *DRB1* 0405-DQB1*0401* and *DRB1*0901-DQB1*0303* haplotypes (ESM Table 7), suggesting that genes outside class II HLA, including class I HLA, contribute to the difference between fulminant and other subtypes of type 1 diabetes.

To further clarify the contribution of genes outside class II HLA to the phenotypic difference between the three subtypes of type 1 diabetes, we genotyped SNPs in an 8.5 Mb region of the extended HLA that ranged from a locus 1.5 Mb telomeric to *HFE* to a locus 0.5 Mb centromeric to *DPB1*. In addition to the association observed in all three subtypes, an association limited to one or two subtypes was also observed, which may contribute to the phenotypic differences between the three disease subtypes. Among these were SNPs in *TAP2* and *DQA2*, which were associated with fulminant, but not with acute-onset or slowly progressive type 1 diabetes, with minor alleles conferring disease susceptibility. In contrast, a SNP in *DOB* was associated with acute-onset and slowly progressive, but not with fulminant, type 1 diabetes, with the minor allele being protective against the disease, as previously reported