Table 3. Concordance of genotype calls between Kapa 2GFast HotStart DNA polymerase and QIAGEN Multiplex PCR Kit.

		Kapa 2G	QIAGEN
1st set	96-plex PCR	99.94% (6,513/6,517 genotype)	
	192-plex PCR	99.89% (7,441/7,449 genotype)	
2nd set	96-plex PCR	99.99% (7,778/7,779 genotype)	
	192-plex PCR	99.99% (7,700/7,701 genotype)	

doi:10.1371/journal.pone.0029967.t003

polymerase and multiplicity in multiplex PCR (192-plex or 96-plex). However, the SNP191, which was amplified by primer pair 191, was successfully genotyped only when the QIAGEN Multiplex PCR Kit was used for the multiplex PCR. The concentration of amplicon amplified by primer pair 99 was the same as the 2.8 nM observed with the amplicon amplified by primer pair 191. SNP99, which was amplified by primer pair 99, was successfully genotyped independently of polymerase type and multiplicity in multiplex PCR (192-plex or 96-plex). These results suggest that the sensitivity in genotyping with Kapa 2GFast HotStart DNA polymerase was lower than the previously used protocol with QIAGEN Multiplex PCR Kit. These results would be explained by a biased amplification with the shortened protocol using Kapa 2GFast HotStart DNA polymerase, which tends to lead to a consequent biased genotyping. However, the investigated number of primer pairs would not be sufficient to decide the sensitivity in genotyping; therefore, it is necessary to continuously accumulate genotyping data. As the investigated number of primer pairs was only 192 (384 primers) in this study, melting temperature of each primer and the number of potential amplicons predicted by the MFE primer software were strongly associated with low sensitivity and low specificity in an amplification, respectively (multiple regression analysis, $P = 1.26 \times 10^{-37}$ and $P = 1.52 \times 10^{-21}$, respectively).

Through the use of Kapa 2GFast HotStart DNA polymerase, the genotype calls for 96 SNPs can be acquired in about 7 hours by the DigiTag2 assay. The genotyping platform with high conversion rate plays an important role for the replication studies to identify the disease associated genes from candidate loci found in the GWAS (genome-wide association study). The DigiTag2 assay with an improved protocol will be an efficient platform for screening an intermediate number of SNPs (tens to hundreds of sites) in the replication studies. Because of limitations in the variation of DNA coded numbers (DCNs), 192-plex genotyping is not available for the current DigiTag2 assay. However, 192-plex PCR can save genomic DNA samples and time for target preparation. Moreover, 192-plex PCR is also available for direct-sequencing and other PCR-based assays to amplify the target regions from genomic DNA.

Supporting Information

Table S1 Sequence information of 192 pairs of locus specific primer.
(XLSX)

Table S2 Results of singleplex PCR with 192 pairs of locus specific primer.
(XLSX)

Table S3 The 15 discordant genotype calls in 8 different conditions.
(XLSX)

Acknowledgments

We would like to thank M. Takasu for technical support, and H. Adachi, N. Tabei and J. Fujimiya (Dynacom Co., Ltd.) for assistance with primer and probe design.

Author Contributions

Conceived and designed the experiments: NN KT. Performed the experiments: YM MS. Analyzed the data: NN YM MS. Contributed reagents/materials/analysis tools: NN YM MS. Wrote the paper: NN KT.

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DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2011; **27**: 844–848.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/dmrr.1234

RESEARCH ARTICLE

Analysis of the HLA and non-HLA susceptibility loci in Japanese type 1 diabetes

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Received: 2 March 2011
Revised: 15 June 2011
Accepted: 16 June 2011

Abstract

Background We previously reported the associations of human leukocyte antigen (HLA) (*DRB1* and *DQB1*), *INS, CTLA4, IL2RA, ERBB3* and *CLEC16A* with Japanese type 1 diabetes (T1D). In this study, we jointly analysed these loci in addition to *IFIH1* and *IL7R*.

Methods A maximum of 790 T1D patients and 953 control subjects were analysed. HLA was determined by sequencing-based typing. Seven non-HLA single nucleotide polymorphisms were genotyped using TaqMan assay.

Results HLA *DRB1*0405*, *DRB1*0901* and *DRB1*0802-DQB1*0302* haplotypes were positively associated with T1D, while the *DRB1*15* haplotypes were negatively associated. Non-HLA single nucleotide polymorphisms, *INS, IL2RA, ERBB3, CLEC16A* and *IL7R* were associated with T1D. By a prediction model using the HLA loci alone (HLA model) or the non-HLA loci alone (non-HLA model), it was revealed that the cumulative effect of the non-HLA model was much weaker than that of the HLA model (average increase in odds ratio: 1.17 *versus* 3.14). Furthermore, the area under the receiver operating characteristic curve of the non-HLA model was also much smaller than that of the HLA model (0.65 *versus* 0.81, $p < 10^{-11}$). Finally, a patient-only analysis revealed the susceptible HLA haplotypes and the risk allele of *INS* to be negatively associated with slower onset of the disease. In addition, the *DRB1*0901* haplotype and the risk alleles of *ERBB3, CLEC16A* and *CTLA4* were positively associated with the co-occurrence of thyroid autoimmunity.

Conclusions Although several non-HLA susceptibility genes in Japanese were confirmed trans-racially and appear to contribute to the heterogeneity of the clinical phenotypes, the cumulative effect on the ability to predict the development of T1D was weak. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords type 1 diabetes; genetic susceptibility; prediction model; single nucleotide polymorphism

Introduction

Human leukocyte antigen (HLA) class II *DRB1* and *DQB1* alleles, or haplotypes, are major susceptibility genes to type 1 diabetes (T1D) in various ethnic groups including Japanese [1–5]. After the HLA class II loci, the second and the third most important loci, with odds ratios of just over 2.0, are the insulin gene (*INS*) and the PTPN22 gene (*PTPN22*) in the Caucasian populations. Furthermore, a number of weak non-HLA susceptibility loci were identified in Caucasians mostly by the recent genome-wide association studies [5,6].

Among Japanese [2,4], the susceptible HLA haplotypes in Caucasians, i.e. DR4 (e.g. *DRB1*0401–DQB1* 0302* and *DRB1*0301–DQB1*0201*), are rare and instead *DRB1*0405–DQB1*0405, DRB1*0802–DQB1*0302* and *DRB1*0901–DQB1*0302* are the major susceptibility haplotypes. In contrast,

protective haplotypes in Japanese are *DRB1*1502–DQB1*0601* in addition to *DRB1*1501–DQB1*0602*, which is also protective in Caucasians. By a multicentre collaboration study group, the Japanese Study Group on Type 1 Diabetes Genetics, the *INS* locus was certainly confirmed to be a non-HLA susceptibility gene [7] and we have also obtained evidence for an association with the *CTLA4* [8], *IL2RA* [9], *ERBB3* [10] and *CLEC16A* [10] loci.

In this study, we jointly analysed the HLA and non-HLA genetic susceptibility genes to evaluate their roles in the prediction of T1D and their effects on the clinical heterogeneity in the Japanese population.

Subjects and methods

Subjects

A total of 1743 Japanese subjects, including 790 patients with T1D and 953 control subjects, were studied. The patients consisted of 441 females and 349 males, with a mean (± standard deviation) age at onset of 30.5 (±17.4) years. An ethics committee from each institute approved the study and informed consent was obtained from all subjects.

Genotyping of HLA class II and non-HLA SNPs

HLA class II *DRB1* and *DQB1* were genotyped using the polymerase chain reaction sequence-specific primer and polymerase chain reaction sequence-specific oligonucleotide methods. The most probable *DRB1–DQB1* haplotypes were deduced from known linkage disequilibria. Seven single nucleotide polymorphisms (SNPs), *INS* rs689, *IL2RA* rs706778, *ERBB3* rs2292239, *CLEC16A* rs2903692, *CTLA4* rs3087243, *IFIH1* rs1990760 and *IL7R* rs6897932, were genotyped using TaqMan assay. Among them, the SNPs of *INS, IL2RA, ERBB3, CLEC16A* and *CTLA4* were previously analysed in the Japanese multicentre collaboration studies [7–10]. The *IFIH1* and *IL7R* nsSNPs, confirmed loci in Caucasians, were added in this present study. Regarding the variations in the other T1D susceptibility loci, *PTPN2* rs2476601, *CCR5* rs333 and *SH2B3* rs3184504, they were not polymorphic in Japanese, and *IL2-IL21* rs2069763, *PTPN2* rs47852 and *CD226* rs763361 were not associated with T1D in the small subset of subjects (~150 patients and ~200 controls; data not shown).

Statistical analyses

The odds ratios for alleles or haplotypes were calculated by a logistic regression analysis after adjusting for sex and age (the age at onset for patients with T1D and age at recruitment for control subjects). Multiplicative gene–gene interactions were evaluated using a logistic regression analysis by including the product of genotypes (coded by 0, 1 and 2) as an interactive term. We constructed a prediction model for T1D using the logistic regression analysis separately for HLA and non-HLA loci. Susceptibility-graded *DRB1–DQB1* genotypes (R/X, N/N, S/N and S/S; S: susceptible, P: protective, N: neutral, X: any) were adopted for the HLA model, while the numbers of risk alleles for the seven SNPs were used for the non-HLA model. The average increases in odds ratio were calculated by a logistic regression analysis after adjusting for sex and age. To evaluate the prediction model, receiver operating characteristic (ROC) curves for the sensitivity and specificity of the prediction model with sex and age were generated and the area under the curve (AUC) was calculated from the ROC curve. The difference in the AUC was evaluated by the method described by Hanley and McNeil [11]. An intra-patient logistic regression analysis was performed in T1D patients to assess the independent role of SNPs in the onset mode (slow onset *versus* acute onset) or co-occurrence of autoimmune thyroid disease (AITD) by including the genotypes of the SNPs simultaneously – sex and age at onset of T1D as variables. The StatsDirect Ver. 2.6.5 (StatsDirect, Cheshire, UK), StatView Ver.5 (SAS Institute, USA) and StatFlex Ver. 6.0 (Artech Co., Ltd., Japan) software programmes were used for these tests. Statistical significance was defined as $p < 0.05$.

Results

HLA haplotypes and non-HLA susceptibility SNPs associated with type 1 diabetes in Japanese

As shown in Table 1, in accord with the previous studies, the HLA *DRB1*0405* (mostly *DRB1*0405–DQB1*0401*), *DRB1*0901* (mostly *DRB1*0901–DQB1*0303*) and *DRB1*0802–DQB1*0302* haplotypes were positively associated with T1D, while *DRB1*15* haplotypes (*DRB1*1501–DQB1*0602* or *DRB1*1502–DQB1*0601*) were negatively associated with the disease. Regarding the seven non-HLA SNPs, the *INS, IL2RA, ERBB3, CLEC16A* and *IL7R* SNPs were significantly associated with T1D. Among these, the associations with *INS* and *ERBB3* were relatively stronger than those of *IL2RA, CLEC16A* and *IL7R*.

Cumulative risk assessment for type 1 diabetes on the basis of HLA *DRB1–DQB1* genotypes, different numbers of risk alleles of the seven non-HLA susceptibility SNPs or both

As there was no evidence of gene–gene interactions, we constructed a prediction model by incorporating either hierarchical HLA genotypes or the number of risk alleles

Table 1. Human leukocyte antigen (HLA) haplotypes and non-HLA susceptibility single nucleotide polymorphisms for the association with type 1 diabetes in Japanese

	Odds ratio	95% Confidence interval	*p* value
HLA			
*DRB1*0405* haplotype	2.7	2.2–3.4	1.2×10^{-18}
*DRB1*0901* haplotype	2.0	1.6–2.5	3.5×10^{-11}
*DRB1*0802* haplotype[a]	3.6	2.0–6.4	1.3×10^{-5}
*DRB1*1501* or **1502* haplotype	0.16	0.11–0.24	1.1×10^{-22}
Non-HLA			
INS rs689	3.6	2.0–6.7	3.2×10^{-5}
IL2RA rs706778	1.2	1.0–1.4	0.020
ERBB3 rs2292239	1.5	1.3–1.8	5.7×10^{-6}
CLEC16A rs2903692	1.2	1.0–1.5	0.033
CTLA4 rs3087243	1.0	0.8–1.1	Not significant
IFIH1 rs1990760	1.1	1.0–1.4	Not significant
IL7R rs6897932	1.3	1.0–1.5	0.018

Adjusted for sex and age by logistic regression, [a]*DRB1*0802–DQB1*0302*.

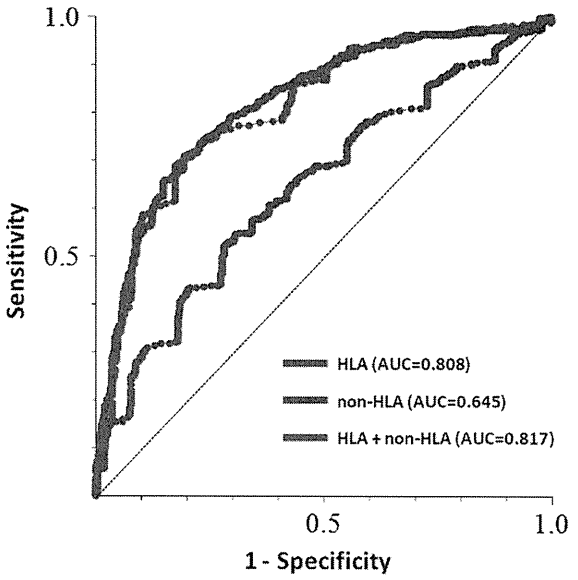

Figure 2. Receiver operating characteristic (ROC) curves for the prediction model on the basis of the human leukocyte antigen (HLA) *DRB1–DQB1* genotypes, the number of risk alleles of the seven non-HLA susceptibility single nucleotide polymorphisms or both. The area under the receiver operating characteristic curve [area under the curve (AUC)] for non-HLA loci was significantly smaller than AUC for HLA loci, $p < 10^{-11}$, while the AUC for both HLA and non-HLA loci was not statistically different from the AUC for HLA loci, $p = 0.773$

for the seven non-HLA loci as an independent variable in addition to sex and age. Although *CTLA4* rs3087243 and *IFIH1* rs1990760 were not significantly associated with T1D (Table 1), we also included these SNPs since the former SNP was significantly associated with the susceptibility to T1D complicated with AITD and the latter SNP was significantly associated with slow onset T1D (data not shown). Figure 1A shows the distribution of the HLA genotypes or the number of non-HLA risk alleles in both patients with T1D and control subjects. The cumulative effect of non-HLA loci (the average increase in odds ratio was 1.17, $p = 0.0007$) was much weaker than that of HLA loci (the average increase in odds ratio was 3.14, $p = 2.34 \times 10^{-42}$). In addition, we estimated the power of prediction by an ROC curve. As shown in Figure 2, the area under the ROC curve (AUC) for the non-HLA loci was also much smaller than that for HLA loci, with values of 0.645 *versus* 0.808, which was highly significant ($p < 10^{-11}$). Furthermore, if we add non-HLA SNPs to the HLA *DRB1–DQB1*, the AUC increases only

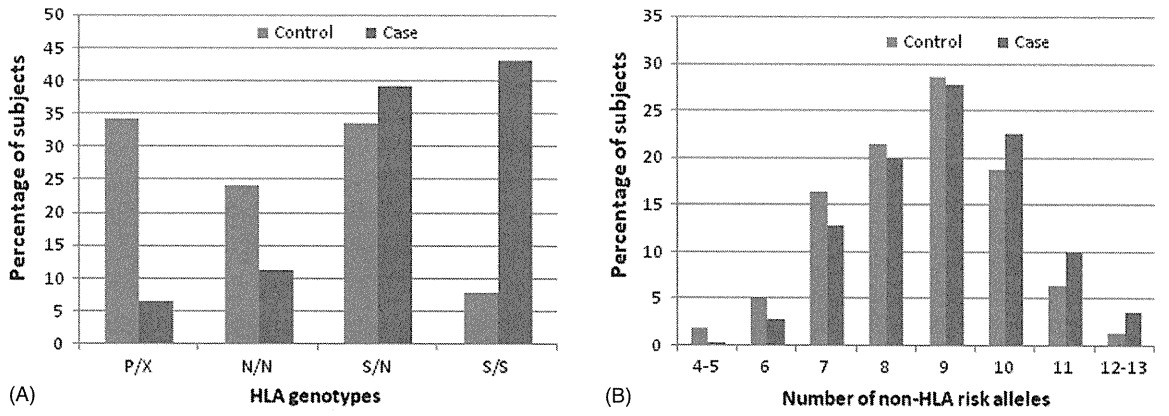

Figure 1. Distribution of the human leukocyte antigen (HLA) genotypes or the number of non-HLA risk alleles in both patients with type 1 diabetes (case) and control subjects (control). (A) HLA *DRB1–DQB1* genotypes (P: protective, S: susceptible, N: neutral, X: any), (B) number of risk alleles of the seven non-HLA susceptibility single nucleotide polymorphisms (*INS* rs689, *IL2RA* rs706778, *ERBB3* rs2292239, *CLEC16A* rs2903692, *CTLA4* rs3087243, *IFIH1* rs1990760 and *IL7R* rs6897932). The average increase in odds ratio was 3.14, $p = 2.34 \times 10^{-42}$ with the hierarchical HLA genotypes, and the average increase in odds ratio was 1.17, $p = 0.0007$ with an increasing number of non-HLA risk alleles

Table 2. Odds ratios for the association with clinical phenotypes among type 1 diabetic patients

	Slow onset *versus* acute onset or fulminant		With autoimmune thyroid disease *versus* without autoimmune thyroid disease	
Variable	Odds ratio	*p* value	Odds ratio	*p* value
Female sex	0.77	NS	3.32	1.6×10^{-5}
Age at onset (years)	1.06	3.0×10^{-12}	1.04	1.9×10^{-5}
*HLA–DRB1*405* haplotype	0.44	0.0002	1.31	NS
*HLA–DRB1*0901* haplotype	0.48	0.0007	1.63	0.029
*HLA–DRB1*0802* haplotype	0.37	0.027	1.28	NS
*HLA–DRB1*15* haplotype	0.82	NS	1.58	NS
INS	0.12	0.014	2.85	NS
IL2RA	0.95	NS	1.39	NS
ERBB3	1.03	NS	1.52	0.048
CLEC16A	1.07	NS	2.06	0.0073
CTLA4	0.81	NS	1.65	0.030
IFIH1	1.37	NS	1.41	NS
IL7R	1.36	NS	0.74	NS

NS, not significant ($p > 0.05$).

a little with a value of 0.817 and the increase is not significant ($p = 0.773$).

Intra-patients analysis for assessing onset mode and AITD co-occurrence

An intra-patient logistic regression analysis was performed to assess the independent role of polymorphisms in the disease phenotypes. We assessed the mode of onset (slow onset *versus* others), and the co-occurrence of AITD, which was defined as Graves' disease, Hashimoto's thyroiditis or positivity for antibodies against thyroid peroxydase (TPO) and/or thyroglobulin. The clinical data on the mode of onset (acute onset, slow onset and fulminant) were available in 440 patients; among them, 313 patients were acute onset, 113 slow onset and 14 fulminant. The definition of the mode of onset was described previously [9]. The clinical data on AITD were available in 309 patients; among them, 124 patients were complicated with AITD and 185 were not. As shown in Table 2, all susceptible HLA haplotypes and the risk allele of *INS* were negatively associated with a slower onset, in addition to an older age at onset. The *DRB1*0901* haplotype, and the risk alleles of *ERBB3, CLEC16A* and *CTLA4* were positively associated with thyroid autoimmunity, in addition to female sex and an older age at onset.

Discussion

In this study, we jointly analysed the HLA and non-HLA susceptibility genes. As shown in Table 1, three susceptible haplotypes and two resistant haplotypes were in accord with previous studies [2,4]. Among the non-HLA SNPs, *INS* rs689 and *ERBB3* rs2292239 were firmly associated with T1D. However, the associations with *IL2RA* rs706778, *CLEC16A* rs2903692 and *IL7R* rs6897932 were weak and the *CTLA4* rs3087243 and *IFIH1* rs1990760 were not significantly associated with whole T1D; further large-scale studies in Japanese will be required to confirm these loci.

Using the susceptibility-graded HLA *DRB1–DQB1* genotypes and the numbers of the risk alleles for the seven SNPs, we constructed a prediction model for T1D. As shown in Figure 1, the cumulative effect of the non-HLA model was much weaker than that of the HLA model when comparing the average increase in odds ratios for each additional haplotype or allele; the AUC score for the ROC curve of the additive genotype scores of the non-HLA SNPs (0.645) was much smaller than that of the HLA genotypes (0.808), and the addition of the non-HLA genetic data resulted in minimal improvement of the prediction by the HLA genotypes. Thus, the performance of the disease risk model for the selected non-HLA SNPs is limited compared to the HLA model. Although we used seven non-HLA loci among over 50 loci identified in Caucasians [6], similar AUC values were reported from the Caucasian genome-wide association studies data, such as AUC scores of 0.81 and 0.65 for the logistic regression model using SNPs with $p < 1 \times 10^{-5}$ and using those outside the MHC region, respectively [12]. Therefore, the relative roles of the HLA and non-HLA components in the genetic architecture of T1D may be similar between Japanese and Caucasians, although there may be non-HLA susceptibility genes unique to Japanese that are yet to be identified.

We next assessed the effects of the HLA and non-HLA genes on disease phenotypes. Regarding the mode of onset, all susceptible HLA haplotypes were negatively associated with slower onset among patients, reflecting the gene dosage effect of the susceptible HLA haplotypes in the onset and progression of disease. In addition, the risk allele of *INS* was found to be negatively associated with a slower onset. This observation is consistent with the association of the protective *INS* allele with preserved beta-cell function reported in Caucasian

T1D [13,14]. We also assessed thyroid autoimmunity frequently complicated in T1D. In addition to the observation already reported in the collaboration study (i.e. an association with *ERBB3, CLEC16A* and *CTLA4* SNPs) [10], the *DRB1*0901* haplotype was found to be associated with AITD. However, the role of this particular HLA haplotype in thyroid autoimmunity is not conclusive, since previous studies failed to detect any shared HLA *DRB1–DQB1* haplotypes between T1D and AITD in Japanese [15,16]. Among the non-HLA *ERBB3, CLEC16A* and *CTLA4* loci, *CLEC16A* and *CTLA4* may be indeed shared genetic loci of immune-related diseases [5,17]. In contrast, the effect of the *ERBB3* locus on other immune-related diseases has been uncertain, but Wang *et al.* has recently reported that ERBB3 may play a critical role in immune regulation by modulating antigen presenting cell function [18].

In conclusion, several non-HLA susceptibility genes appear to contribute to the heterogeneity of the clinical phenotypes of T1D patients, although their cumulative effect on the ability to predict the development of T1D is much weaker than that of HLA. Further studies, such as genome-wide association studies and studies of rare disease-associated variants, are called for.

Acknowledgements

We thank Ritsuko Doki for her valuable assistance. The members of the Japanese Study Group on Type 1 Diabetes Genetics are Takuya Awata, Hiroshi Ikegami, Eiji Kawasaki, Tetsuro Kobayashi, Taro Maruyama, Koji Nakanishi, Akira and Shimada and Kazuma Takahashi. This work was supported in part by a grant-in-aid for 'Support Project of Strategic Research Center in Private Universities' from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to Saitama Medical University Research Center for Genomic Medicine.

Conflict of interest

None declared.

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DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2011; **27**: 895–898.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/dmrr.1269

RESEARCH ARTICLE

Zinc transporter 8 autoantibodies in fulminant, acute-onset, and slow-onset patients with type 1 diabetes

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Received: 25 February 2011
Revised: 3 June 2011
Accepted: 8 June 2011

Abstract

Background The aim of this study was to determine the prevalence and role of autoantibodies to zinc transporter 8 (ZnT8A) in three forms (fulminant, acute-onset, and slow-onset) of Japanese patients with type 1 diabetes.

Methods One-hundred and ninety-six new-onset patients with type 1 diabetes were studied: 85 were fulminant, 81 acute-onset, and 30 slow-onset type 1 diabetes. ZnT8A were determined by radioimmunoassay using a hybrid ZnT8 carboxy-terminal construct (aa268-369) carrying 325Trp and 325Arg. Furthermore, ZnT8A epitopes were analysed using ZnT8 constructs incorporating the known aa325 variants (Trp, Arg, and Gln).

Results ZnT8A were detected in 58% patients with acute-onset and 20% with slow-onset type 1 diabetes ($p < 0.0005$). In contrast, none of sera from fulminant type 1 diabetes were reactive to ZnT8 construct. Conversion of Arg or Trp to Gln at aa325 abolished reactivity in 59% of patients with an age of onset >10 years, which was significantly higher than that in patients ≤10 years of age (33%, $p < 0.05$).

Conclusions These results suggest that ZnT8A are an additional useful marker for acute-onset type 1 diabetes, but not a diagnostic marker for fulminant type 1 diabetes, and ZnT8A epitope recognition is different according to the onset age. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords Japanese; type 1 diabetes; fulminant; slow-onset; ZnT8; autoantibodies

Background

Growing evidence has accumulated that there are at least three subtypes of type 1 diabetes in Japan, acute-onset 'classical', slow-onset, and fulminant type 1 diabetes [1]. Patients with slow-onset type 1 diabetes are detected by urine glucose screening at healthy check with minimal or no clinical symptoms, and require insulin treatment within 2 years of the initial positive urine glucose test [2]. Fulminant type 1 diabetes is characterized by extremely rapid onset and fulminant symptoms, including marked hyperglycaemia and severe diabetic ketoacidosis with normal-to-near normal HbA1c levels [1]. In childhood- and adolescent-onset type 1 diabetes, about 90% have the 'classical' form, and the remainders belong to the slow-onset form. Fulminant type 1 diabetes is rare in the childhood. Conversely, about two-thirds of patients with adult-onset diabetes have the slow-onset form, and ~20% of those with ketosis or ketoacidosis fall into the category of fulminant type 1 diabetes [1]. Circulating anti-islet autoantibodies distinguish type 1A diabetes from other diabetics and are now established markers for the

clinical diagnosis and the preclinical phase of this disease. However, anti-islet autoantibodies are merely detected in patients with fulminant type 1 diabetes [3].

The zinc transporter 8 (ZnT8) is a recently identified autoantigen in type 1 diabetes localized to the insulin granule of the pancreatic β cell. Previous studies have reported that the humoral autoreactivity to ZnT8 is found in 60–80% of patients with type 1 diabetes [4,5]. The aim of this study was to evaluate the prevalence of ZnT8A in three forms of type 1 diabetes and establish their potential use as additional marker of autoimmunity and phenotype characterization in the Japanese population.

Subjects and methods

Subjects

A total of 196 patients with type 1 diabetes of Japanese origin were enrolled in this study. Sera from 13 fulminant, 81 acute-onset (females 63.0%, age of onset 19.1 ± 14.5 years), and 30 slow-onset (females 56.7%, age of onset 29.7 ± 18.0 years) type 1 diabetes were consecutively recruited at our hospital between 1982 and 2008. The remaining 72 sera from fulminant type 1 diabetes were provided from the Fulminant Type 1 Diabetes Committee of the Japan Diabetes Society [3]. Therefore, a total of 85 patients with fulminant type 1 diabetes (females 36.5%, age of onset 43.0 ± 16.1 years) were studied. All patients with type 1 diabetes analysed in the present study were diagnosed in accordance with the American Diabetes Association criteria for the classification of diabetes [6]. All sera were obtained within 2 weeks after the initiation of insulin treatment. All subjects were informed of the purpose of the study, and their consent was obtained. Protocols were approved by the ethics committee of the Nagasaki University and the Japan Diabetes Society. Sera were stored at −20 °C until use.

ZnT8 autoantibody assay

ZnT8A were determined by radioligand binding assay using the a fusion cDNA construct of the carboxy-terminal domains (aa268–369) carrying 325Trp and 325Arg (CW-CR) as described previously [7]. The cut-off index for ZnT8A-CW-CR was an index of 0.007, which was based on the 99th percentile of sera from 139 healthy control subjects. In this study, ZnT8A were considered as 'positive' if sera were ranked as positive for ZnT8A-CW-CR. In the DASP2009, this assay had 40% sensitivity and 100% specificity. Autoantibody reactivities to ZnT8 aa325 variants were also determined using the carboxy-terminal domains (aa268–369) of cDNA carrying either 325Trp, 325Arg, or 325Gln to analyse an epitope specificity. The cut-off index was an index of 0.018 for ZnT8A-325Trp, 0.016 for ZnT8A-325Arg, and 0.006 for ZnT8A-325Gln based on the 99th percentile of sera from 139 healthy control subjects. Autoantibodies to ZnT8 aa325 variants were determined in 85 fulminant, 76 acute-onset, and 30 slow-onset patients with type 1 diabetes because of the serum availability.

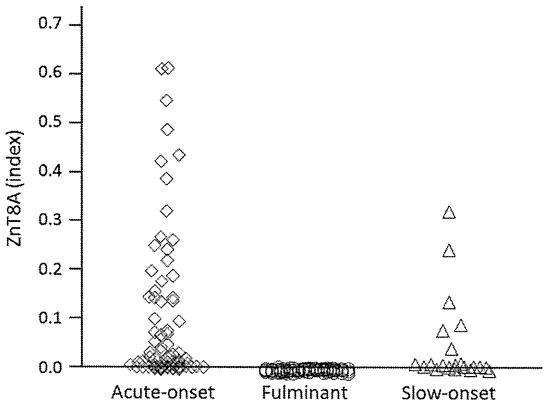

Figure 1. The ZnT8A titres in patients with three forms of type 1 diabetes. The prevalence of ZnT8A in patients with acute-onset, fulminant, and slow-onset type 1 diabetes was 58, 0, and 20%, respectively

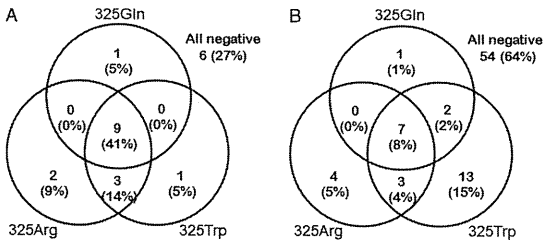

Figure 2. Relationship between autoantibody responses to 325Trp, 325Arg, and 325Gln constructs and age of diabetes onset in patients with acute- and slow-onset type 1 diabetes. (A) Age of onset ≤10 years ($n = 22$); (B) age of onset >10 years ($n = 84$)

Statistical analysis

Results were expressed as mean ± standard deviation unless otherwise indicated. Autoantibody prevalence was compared using the Chi-squared test and Fisher's exact test where appropriate. A p-value less than 0.05 was considered statistically significant.

Results

ZnT8A in three forms of type 1 diabetes

ZnT8A (ZnT8A-CW-CR) were detected in 47/81 (58%) patients with acute-onset and 6/30 (20%) with slow-onset type 1 diabetes at disease onset. However, none of 85 patients with fulminant type 1 diabetes were positive for ZnT8A (Figure 1). The prevalence of ZnT8A in patients with acute-onset was significantly higher than that in patients with slow-onset whose diabetes was diagnosed on the basis of urine glucose screening ($p < 0.0005$). In

acute- and slow-onset type 1 diabetes, the prevalence of ZnT8A was significantly higher in subjects with an age of onset ≤ 10 years (17/22, 77%) than in patients >10 years of age (36/89, 40%, $p < 0.005$).

Autoantibody reactivity to ZnT8 aa325 variants

Sera were also tested for the reactivity to the carboxy-terminal ZnT8 constructs bearing 325Trp, 325Arg, or 325Gln. None of 85 patients with fulminant type 1 diabetes reacted with any ZnT8 variant constructs. Forty-six of 106 (43%) patients with acute- and slow-onset type 1 diabetes reacted to at least one construct, with the highest response recorded in reaction to the 325Trp construct (36%) followed by 325Arg (26%) and 325Gln (19%) constructs. Analysis of the overlap in responses shows that 6 and 13% of patients react to the 325Arg or 325Trp construct alone and rarely to 325Gln alone; 15% of patients reacted to all three constructs. This indicated that for these individuals who reacted to all variants, the amino acid at position 325 was not a determination of autoantibody reactivity.

The relationship between autoantibody reactivities to ZnT8 variant constructs and age of diabetes onset is illustrated in Figure 2A and B. The prevalence of ZnT8A measured with either 325Trp, 325Arg, or 325Gln construct in subjects with an age of onset ≤10 years was significantly higher than that in patients >10 years of age ($p < 0.05$). Furthermore, the prevalence of patients reacted to all three constructs was significantly higher in the younger onset group compared with the older onset group (41 *versus* 8%, $p < 0.0005$). Conversion of Arg or Trp to Gln at aa325 abolished reactivity for 23 of 39 patients (59%) in the older age group, which was higher than that in the younger age group (33%, $p < 0.05$).

Conclusions

This study shows that (1) ZnT8A are an additional useful marker for acute-onset type 1 diabetes, (2) ZnT8A reactivity differs between younger and older onset cases, and (3) ZnT8A are not a diagnostic marker for fulminant type 1 diabetes. ZnT8A were detected in 58% patients with acute-onset type 1 diabetes, which is significantly higher than that in slow-onset type 1 diabetes (20%) (Figure 1). Thus, ZnT8A identify heterogeneity in the mode of diabetes onset and are apparently markers of acute-onset type 1 diabetes. Similar to IA-2A [2], ZnT8A were prevalent in younger individuals in the Japanese population, emphasizing the utility of ZnT8A as an additional marker in younger subjects.

We and others recently reported that the amino acid encoded by a common polymorphism in human ZnT8 at aa325 (rs13266634 and Arg325Trp) is a key determinant of two major conformational epitopes in the protein [7,8]. Furthermore, Wenzlau and coworkers reported that ZnT8 carboxy-terminal domain contains at least two distinct epitopes, one of which is critically dependent on the presence of Arg or Trp at position 325 [9]. The proportion of individuals who precipitated both the wild-type and the mutant constructs was higher in younger onset patients compared with older onset patients, suggesting that non-restricted ZnT8A epitope is associated with early development of disease. Further studies in the pre-diabetic phase are required to support this suggestion.

Finally, we found for the first time that ZnT8A are not a diagnostic marker for fulminant type 1 diabetes, which is a subtype of type 1 diabetes characterized by extremely rapid onset with nearly normal HbA1c level, frequent flu-like symptoms just before the disease onset, and virtually no C-peptide secretion at disease onset [3]. In conclusion, our present data suggest that ZnT8A are an additional useful marker for acute-onset type 1 diabetes, but not a diagnostic marker for fulminant type 1 diabetes, and ZnT8A epitope recognition is different according to the onset age.

Acknowledgements

This study was partly supported by a grant from the Ministry of Education, Culture, Science, Sports and Technology of Japan. We thank the Fulminant Type 1 Diabetes Committee of the Japan Diabetes Society for providing precious sera from fulminant type 1 diabetes. Vector pJH4.1 and BUN-E antiserum were kindly provided by Dr. John Hutton, Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO, USA.

Conflict of interest

The authors have no conflicts of interest.

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Type 1 Diabetes and Interferon Therapy

A nationwide survey in Japan

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OBJECTIVE—Interferon therapy can trigger induction of several autoimmune diseases, including type 1 diabetes. To assess the clinical, immunologic, and genetic characteristics of type 1 diabetes induced by interferon therapy, we conducted a nationwide cross-sectional survey.

RESEARCH DESIGN AND METHODS—Clinical characteristics, anti-islet autoantibodies, and HLA-DR typing were examined in 91 patients for whom type 1 diabetes developed during or shortly after interferon therapy.

RESULTS—Median age at the onset of type 1 diabetes was 56 (interquartile range 48–63) years and mean ± SD BMI was 20.8 ± 2.7 kg/m^2. The time period from the initiation of interferon therapy to type 1 diabetes onset in patients receiving pegylated interferon and ribavirin was significantly shorter than that in patients with nonpegylated interferon single therapy ($P < 0.05$). Anti-islet autoantibodies were detected in 94.5% of patients at diabetes onset. Type 1 diabetes susceptibility HLA-DRs in the Japanese population, DR4 and DR9, were also associated with interferon treatment–related type 1 diabetes. Furthermore, the prevalence of HLA-DR13 was significantly higher in interferon treatment–related type 1 diabetes than in healthy control subjects (odds ratio 3.80 [95% CI 2.20–7.55]; $P < 0.0001$) and classical type 1 diabetes (2.15 [1.17–3.93]; $P < 0.05$).

CONCLUSIONS—Anti-islet autoantibodies should be investigated before and during interferon therapy to identify subjects at high risk of type 1 diabetes. Stronger antiviral treatment may induce earlier development of type 1 diabetes. Furthermore, patients who develop interferon-induced type 1 diabetes are genetically susceptible.

Diabetes Care 34:2084–2089, 2011

Interferons are ubiquitous cytokines produced by all mononuclear cell types as part of an immune response to a viral infection or other immune trigger; they induce antiviral proteins and activate natural killer cells (1). Interferon-based treatment is currently used in a number of conditions, including chronic viral hepatitis, hematological malignancies such as chronic myelogenous leukemia, renal cell carcinoma, and melanoma. On the other hand, the development of autoimmune diseases, such as autoimmune thyroid disease (AITD), autoimmune hemolytic anemia, and Behçet disease, has been reported as a side effect during or after treatment with interferon (2); type 1 diabetes has also been reported sporadically (3–8). Conversely, there has been a report that islet autoantibodies did not appear in patients with interferon treatment (9). Also, there is a single case report in which diabetes was resolved when interferon treatment stopped (10). Because the number of reports on interferon treatment–related type 1 diabetes have increased in recent years, it is important for preventative purposes to investigate the clinical, immunologic, and genetic characteristics of such cases to identify the predictive markers. In the current study, we conducted a nationwide survey of patients with type 1 diabetes that developed during or after interferon therapy under the auspices of the Japan Diabetes Society.

RESEARCH DESIGN AND METHODS

—We asked all the members of the Japan Diabetes Society through direct mail and the readership of the *Journal of the Japan Diabetes Society* whether they knew of candidates who had developed type 1 diabetes during or after interferon treatment. We received 48 positive responses. To those who responded positively, we sent questionnaires asking for a description of their characteristics, including the disease for which interferon-based treatment was administered, the type of interferon used, the use of ribavirin combination therapy, the co-occurrence of AITD, and the number of patients with type 1 diabetes and interferon-treated patients in their institutions. We received data on 62 cases. In addition, we searched the PubMed/Medline and Ichushi-Web (Japan Medical Abstract Society) databases for the period 1983 to 2010 using the keywords interferon, type 1 diabetes, and Japanese and identified 29 additional cases that did not overlap with the previous 62 cases. Therefore, a total of 91 patients (male, 48; female, 43) of Japanese origin were

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Received 4 December 2010 and accepted 1 June 2011.
DOI: 10.2337/dc10-2274
This article contains Supplementary Data online at http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc10-2274/-/DC1.
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investigated in this study. Patients were reported from regions throughout Japan, from Hokkaido Island to Kyushu Island, and although the majority of cases were clustered in highly populated areas they were not restricted to any specific region or prefecture in Japan.

Type 1 diabetic patients were divided into three groups according to the mode of diabetes onset (acute onset, slow onset, and fulminant). Among the following five criteria, patients who met either criteria 1–4 or criteria 1–3 plus criterion 5 were placed in the acute-onset type 1 diabetes group: *1*) the presence of ketosis or ketoacidosis at the onset of diabetes; 2) the presence of hyperglycemic symptoms for <3 months before the commencement of insulin therapy; 3) the requirement of insulin replacement therapy at both onset and 6 months after onset; *4*) the presence of at least one anti-islet autoantibody (GAD autoantibody [GADAb], islet cell antibody [ICA], insulin autoantibody [IAA], or insulinoma-associated antigen-2 autoantibody [IA-2Ab]); and 5) decreased insulin-secreting capacity (urinary C-peptide excretion <20 μg/day, fasting serum C-peptide level <0.4 ng/mL, or peak serum C-peptide level <1.0 ng/mL after glucagon injection or meal load). Diagnosis of slow-onset type 1 diabetes was based on the following criteria: *1*) originally diagnosed as type 2 diabetes and no sign of ketosis at diabetes onset; 2) proven anti-islet autoantibody positivity; and 3) insulin treatment started ≥12 months after the diagnosis. The patients who met the following criteria were placed in the fulminant type 1 diabetes group: *1*) ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms; 2) plasma glucose levels ≥16 mmol/L and HbA_{1c} <8.9% at the first visit, and 3) urinary C-peptide level <10 μg/day, fasting serum C-peptide level <0.3 ng/mL, or serum C-peptide <0.5 ng/mL after glucagon or a meal load (11).

AITD was defined as Graves disease, Hashimoto thyroiditis, or the presence of autoantibodies to thyroid peroxidase, thyroglobulin, or thyrotropin receptor.

Autoantibodies

GADAb, ICA, IA-2Ab, and IAA were determined at the onset of type 1 diabetes. GADAb, IA-2Ab, and IAA were measured by radioimmunoassay or radioligand-binding assay, and ICAs were measured by immunohistochemical methods (12). The disease sensitivity/specificity for GADAb, IA-2Ab, and IAA was 82.6/93.6, 66.0/98.9, and 32.0/98.9%, respectively, in the Diabetes Autoantibody Standardization Program 2009.

HLA typing

The serological subtype of HLA-DR or DRB1 genotype was determined (13,14). Allele frequencies were obtained by direct counting. As a control for the analysis of HLA-DR, we also studied 304 unrelated healthy individuals (115 females; median age 45.0 years [range 20.0–74.0]) and 192 patients with classical type 1 diabetes (122 females; age at onset 27.0 years [1.0–75.0]).

Statistical analysis

The results are given as median (interquartile range) or mean ± SD unless otherwise indicated. Statistical analysis was performed with a χ^2 test and the Mann-Whitney *U* test. The multiple comparison test was used to compare the groups with regard to the duration from initiation of interferon therapy to type 1 diabetes onset. The cumulative free incidence rate of type 1 diabetes was compared using the Kaplan-Meier method with log-rank test. The significance of differences in the distribution of HLA-DR alleles between case and control subjects was determined by a χ^2 test. Odds ratios (ORs) (95% CI) were also calculated. StatView (version 5.0; SAS Institute, Cary, NC) were used for these tests. A *P* value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics at type 1 diabetes onset

Table 1 summarizes the clinical characteristics of 91 patients with type 1 diabetes developed during or after interferon therapy at diabetes onset. The female-to-male ratio was 0.90, which is lower than that for classical type 1 diabetes in the Japanese

Table 1—*Clinical characteristics of 91 patients with interferon treatment–related type 1 diabetes*

Sex (male)	48 (53.4)
Age at onset of type 1 diabetes (years)	56 (48–63)
Family history of type 1 diabetes	0 (0)
Family history of type 2 diabetes	23 (25.3)
Past history of type 2 diabetes	14 (15.4)
Thirst	69 (75.8)
Body weight loss	56 (61.5)
Indication for IFN therapy	
Chronic HCV	85 (93.4)
Chronic hepatitis B	1 (1.1)
Chronic myelogenic leukemia	2 (2.2)
Renal cell carcinoma	3 (3.3)
HCV genotype (*n* = 32)	
1a	0 (0)
1b	26 (81.3)
2a	3 (9.4)
2b	3 (9.4)
Type of IFN	
IFNα	10 (9.9)
IFNα-2a	11 (12.1)
PegIFNα-2a	8 (8.8)
IFNα-2b	21 (23.1)
PegIFNα-2b	38 (41.8)
IFNβ	3 (3.3)
Combination of ribavirin (from 2002)	52 (72.2)
Mode of type 1 diabetes	
Fulminant onset	5 (5.5)
Acute onset	74 (81.3)
Slow onset	7 (7.7)
Others	5 (5.5)
Period from recent IFN treatment to type 1 diabetes onset (years)	0.68 (0.38–1.75)
Co-occurrence of autoimmune thyroid disease	25 (27.5)

Data are *n* (%) or median (interquartile range). Family history of type 1 or type 2 diabetes was given for first-degree relatives. Body weight loss was defined as losing at least 5% of usual body weight within a few months. IFN, interferon; IFNα, natural IFNα.

population (1.4–1.5) (11). Median age at onset of type 1 diabetes was 56 years (interquartile range 48–63). Fourteen (15.4%) case subjects had been diagnosed as having type 2 diabetes many years before the diagnosis of type 1 diabetes. Thirst and body weight loss were observed in 75.8 and 61.5% of patients, respectively, and the mean weight loss rate was 15.8 ± 25.4%. Interferon had been administered for chronic hepatitis C virus (HCV) in 85, for chronic hepatitis B in 1, for chronic myelogenic leukemia in 2, and for renal cell carcinoma in 3 patients. HCV genotypes were analyzed in 32 cases. Genotypes 1a, 1b, 2a, and 2b were detected in 0, 26 (81.3%), 3 (9.4%), and 3 patients (9.4%), respectively.

There are at least three subtypes of type 1 diabetes based on their mode of onset, i.e., acute onset, slow onset, and fulminant onset, in Japanese patients. Seventy-four of 91 patients (81.3%) were classified as having the acute onset form, 7 (7.7%) the slow onset form, and 5 (5.5%) the fulminant onset form, with the remaining 5 unclassified. The median period from recent interferon treatment to type 1 diabetes onset was 0.68 years (interquartile range 0.38–1.75; range 0.02–9.62). The co-occurrence of AITD was observed in 25 cases (27.5%).

The total number of patients with type 1 diabetes and of interferon-treated patients in the institutions from which questionnaires had been returned were 5,264 and 18,110, respectively. Therefore, the proportion of cases with interferon treatment–related diabetes among patients with type 1 diabetes was estimated to be 1.18%. Furthermore, the prevalence of interferon treatment–related type 1 diabetes among interferon-treated patients was estimated to be 0.34%, which is 10 times higher than that in the general population (~0.03%) (11). These results suggest that interferon treatment is associated with the development of type 1 diabetes.

Period from initiation or termination of interferon treatment to type 1 diabetes onset

Figure 1 summarizes the mean period from initiation of interferon treatment to the development of type 1 diabetes. Sixty-four patients developed type 1 diabetes during interferon treatment and were divided into four groups based on the type of interferon (nonpegylated interferon [non-PegIFN] or pegylated interferon [PegIFN]) and the use of ribavirin. Compared with the non-PegIFN single-therapy group (median 0.64 years [interquartile range 0.32–1.23; range 0.02–9.01]), the median period was significantly shortened in the PegIFN plus ribavirin group (0.45 [0.29–0.71; 0.17–1.06]; $P < 0.05$). Furthermore, 92.9% (39 of 42) of patients treated with PegIFN or ribavirin developed type 1 diabetes within 1 year, which was significantly higher than the incidence in the non-PegIFN single-therapy group (66.7% [14 of 21]; $P < 0.05$). Kaplan-Meier analysis also revealed a faster development of type 1 diabetes in the PegIFN or ribavirin group compared with that in the non-PegIFN single-therapy group ($P < 0.05$ by log-rank test) (Supplementary Fig. 1).

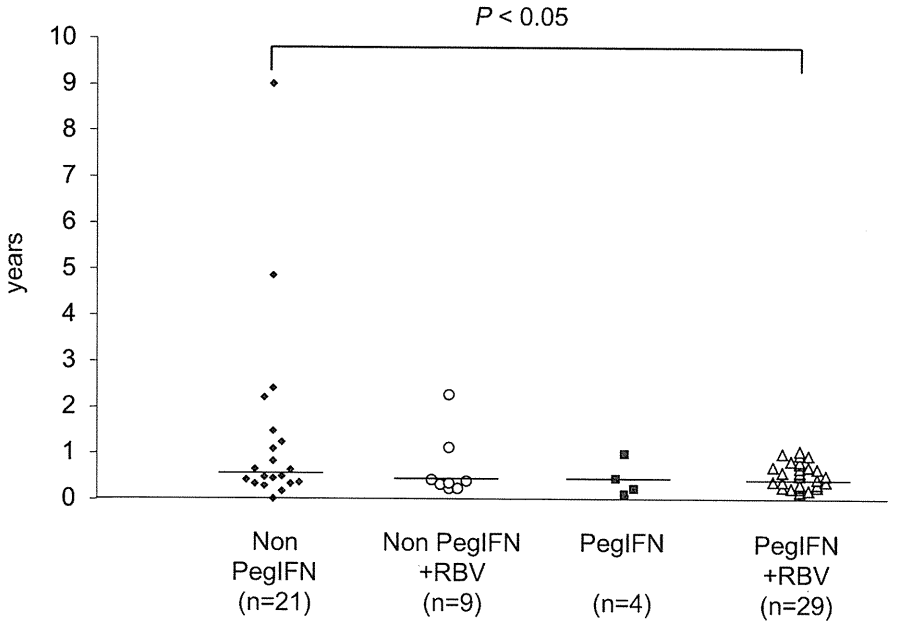

Figure 1—*Period from initiation of interferon treatment to type 1 diabetes onset. RBV, ribavirin. Data were unavailable for 1 of the 64 patients who developed type 1 diabetes during interferon therapy. The horizontal bar indicates the median year of interferon treatment in each group.*

Twenty-six patients (28.6%) developed type 1 diabetes after interferon treatment was terminated, and the median period from the termination of interferon treatment to the onset of type 1 diabetes was 0.75 (range 0.19–1.62) years. The data on the relation between type 1 diabetes development and the initiation or termination time of the interferon treatment were unavailable in the remaining one patient.

Laboratory findings at type 1 diabetes onset

Table 2 summarizes the characteristics of patients before interferon therapy and at type 1 diabetes onset. Although the positivity of HCV RNA was 98.5% at the initiation of interferon treatment, it was 40.6% at type 1 diabetes onset. Therefore, ~60% of patients turned HCV RNA negative before the diagnosis of type 1 diabetes. It is of interest that the incidence of those who were HCV RNA negative was significantly lower in female patients (39.3%) compared with that in male patients (75.0%; $P < 0.005$). Furthermore, among female patients, the period of interferon treatment before the development of type 1 diabetes was significantly longer in patients with HCV RNA (114.8 ± 123.0 weeks) than in HCV RNA–negative patients (36.4 ± 17.5 weeks; $P < 0.05$). No associations were observed between the period of interferon treatment before the development of type 1 diabetes and HCV RNA positivity among male patients (data not shown).

Mean plasma glucose, HbA_{1c} levels, and the positivity of urinary ketone body were 360.7 ± 130.1 mg/dL, 10.5 ± 2.5%, and 66.2%, respectively. The mean values of daily urinary C-peptide excretion, fasting serum C-peptide concentrations, and daily insulin dose were 28.8 ± 28.8 μg/day, 1.0 ± 1.7 ng/mL, and 0.54 ± 0.30 units/kg/day, respectively. The frequencies of GADAb and IA-2Ab were 92.9 and 22.6%, respectively, and 94.5% of patients were positive for at least one of GADAb, ICA, IA-2Ab, or IAA. In comparison with the previously reported frequency of GADAb in patients with idiopathic fulminant type 1 diabetes (15), the frequency

Table 2—*Characteristics of patients before interferon therapy and at type 1 diabetes onset*

	Before interferon therapy	At type 1 diabetes onset	*P*
n	91	91	
BMI (kg/m^2)	24.1 ± 3.5	20.8 ± 2.7	<0.0001
Fasting plasma glucose (mg/dL)	104.3 ± 19.7	360.7 ± 130.1	<0.0001
Serum HCV RNA (positive/negative)	65/1	26/38	<0.0001
Aspartate transaminase (IU/L)	59.1 ± 32.2	36.2 ± 24.4	<0.0001
Alanine transaminase (IU/L)	71.7 ± 43.7	40.6 ± 35.6	<0.0001
Hemoglobin (g/dL)	14.4 ± 1.3	12.9 ± 1.8	<0.0001
Platelets (×10^4/μL)	17.2 ± 7.3	12.8 ± 4.7	<0.0001
HbA_{1c} (%)*	6.5 ± 1.5	10.5 ± 2.5	<0.0001
Glycoalbumin (%)	N.D.	38.4 ± 13.1	
Urine ketone body (positive/negative)	0/21	43/22	<0.0001
Plasma ketone body (μmol/L)	N.D.	3,166.9 ± 2,751.5	
Urinary C-peptide (μg/day)	N.D.	28.8 ± 28.8	
Fasting serum C-peptide (ng/mL)	N.D.	1.0 ± 1.7	
Stimulated serum C-peptide (ng/mL)	N.D.	1.4 ± 1.8	
Anti-islet autoantibodies (positive/negative)	N.D.	86/5	
GADAb (positive/negative)	N.D.	79/6	
GADAb (units/mL)	N.D.	14,103.9 ± 32,213.5	
IA-2Ab (positive/negative)	N.D.	7/24	
ICA (positive/negative)	N.D.	9/7	
IAA (positive/negative)	N.D.	2/4	
Dose of daily insulin (units/kg/day)	N.D.	0.54 ± 0.30	

Data are means ± SD or *n* positive/*n* negative. Data for anti-islet autoantibodies are positive if patients have at least one of GADAb, ICA, IA-2Ab, or IAA. HbA_{1c} (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP). N.D., not determined. *Fourteen patients with type 2 diabetes who had been diagnosed before the initiation of interferon therapy are included.

of GADAb was higher (3 of 5; 60%) in interferon treatment–related patients with fulminant type 1 diabetes. Furthermore, GADAb emerged in 2 of 85 patients after the onset of type 1 diabetes. The mean GADAb levels were 14,103.9 ± 32,213.5 units/mL (range 0 to 235,000) and were not associated with the presence or absence of AITD, although extremely high levels of GADAb have been reported in patients with type 1 diabetes and AITD (16) (Supplementary Fig. 2).

HLA-DR allele frequency

Table 3 summarizes the frequencies of the HLA-DR allele in patients with interferon treatment–related type 1 diabetes, classical type 1 diabetes, and healthy control subjects. The frequencies of HLA-DR4, -DR9, and -DR13 were significantly higher in interferon treatment–related type 1 diabetes, whereas those of HLA-DR8 and -DR15 were significantly lower than those in control subjects. The HLA-DR13 allele was observed in 15.4% of interferon treatment–related type 1 diabetic patients but in 7.8% of classical type 1 diabetic patients, with a significant difference in the frequency of HLA-DR13 between these two groups (OR 2.15 [95% CI 1.17–3.93], $P < 0.05$). The frequency of HLA-DR14 was also significantly higher than that in patients with classical type 1 diabetes (14.2 [3.03–66.7], $P < 0.0001$).

CONCLUSIONS—In the current study, we investigated the clinical, immunologic, and genetic characteristics of Japanese patients with type 1 diabetes that developed during or shortly after interferon therapy. This was the first study to investigate the mode of type 1 diabetes onset in this type of diabetes. The median period from the initiation of interferon treatment to type 1 diabetes was found to be short (~0.7 years), and the majority of patients showed the abrupt-onset form, suggesting rapid β-cell destruction. Among the patients who developed type 1 diabetes after 2002, ribavirin was used in 72.7% of cases and in 75.4% of the patients who developed type 1 diabetes after 2004, when PegIFN began to be covered by health insurance in Japan, PegIFN was used. Moreover, when the period to the development of type 1 diabetes based on the type of interferon (non-PegIFN or PegIFN) and the use of ribavirin were compared, the length of time was significantly shorter in the PegIFN plus ribavirin group (Fig. 1), suggesting that deviation to the Th1-type immune response by ribavirin (17) and extension of the half-life of interferon by pegylation (18) may be among the causes of the increased number of type 1 diabetic patients.

Anti-islet autoantibodies, especially GADAb, were highly detected at the onset of type 1 diabetes. Furthermore, the seroconversion of GADAb during interferon treatment has been reported in ~40% of patients with chronic viral hepatitis who developed type 1 diabetes (19). These findings suggest that measurement of anti-islet autoantibodies before and during interferon treatment is useful for identifying subjects at high risk of developing type 1 diabetes. The sequential study of anti-islet autoantibodies is required to determine the interval from the emergence of anti-islet autoantibodies to the development of type 1 diabetes and possibly to ascertain an effective and economical measurement frequency as early predictive markers.

A genetic predisposition is necessary but not sufficient for the development of type 1 diabetes. To date, there has been no reported investigation of the genetic factors in interferon treatment–related type 1

Table 3—*HLA-DR allele frequency in patients with interferon treatment–related type 1 diabetes, classic type 1 diabetes, and healthy control subjects*

	Interferon treatment–related type 1 diabetic patients	Classical type 1 diabetic patients	Control subjects	Interferon treatment vs. control			Interferon treatment vs. classical type 1 diabetes		
				P	OR	95% CI	*P*	OR	95% CI
n	130	384	608						
DR1	4.6 (6)	2.1 (8)	5.6 (34)						
DR4	34.6 (45)	38.0 (146)	22.4 (136)	<0.01	1.84	1.22–2.77			
DR8	7.7 (10)	9.9 (38)	15.3 (93)	<0.05	0.46	0.23–0.91			
DR9	26.9 (35)	29.4 (113)	16.4 (100)	<0.01	1.87	1.20–2.92			
DR12	1.5 (2)	3.4 (13)	5.1 (31)						
DR13	15.4 (20)	7.8 (30)	4.3 (26)	<0.0001	3.80	2.20–7.55	<0.05	2.15	1.17–3.93
DR14	6.9 (9)	0.5 (2)	8.6 (52)				<0.0001	14.2	3.03–66.7
DR15	2.3 (3)	4.7 (18)	17.8 (108)	<0.0001	0.08	0.03–0.35			
Others	0 (0)	4.2 (16)	4.6 (28)						

Data are % (*n*) unless otherwise indicated.

diabetes. It has been reported that the genetic background of Japanese type 1 diabetes differs from that of Caucasians. The major susceptible class II HLA antigens in Japanese classical type 1 diabetes are DR4 and DR9 (11). In the current study, we have demonstrated the higher frequency of HLA-DR13 in interferon treatment–related type 1 diabetes compared with that in classical type 1 diabetic and healthy control subjects (Table 3). Furthermore, no differences in *HLA-DR13 frequency have been reported between healthy control subjects and patients with chronic HCV in the Japanese population (20). These results highlight the differences in the contribution of HLA-DR subtypes to susceptibility to interferon treatment–related and classical type 1 diabetes and the importance of HLA-DR13 in influencing the progression of type 1 diabetes during or after interferon therapy.

The pathogenesis of type 1 diabetes in response to interferon treatment remains unclear. However, evidence is accumulating regarding the association between type 1 interferon and type 1 diabetes. The overexpression of interferon-α in the pancreases of patients with type 1 diabetes (21) and a preventative effect of type 1 diabetes by interferon-α–neutralizing antibody in transgenic mice in which the β-cells express interferon-α have been reported (22). Furthermore, interferon-α is known to increase major histocompatibility complex class I antigen expression on cell membranes and to activate T cells and natural killer cells (23). These findings are in agreement with the hypothesis that interferon-α is involved in the development of type 1 diabetes in humans.

Our study has limitations. We did not assess whether anti-islet autoantibodies were induced in interferon-treated patients who did not develop type 1 diabetes. We also did not examine the frequencies of HLA-DR alleles. Fabris et al. (24) have reported that the prevalence of markers of anti-islet autoimmunity in HCV-positive patients increased during interferon-α therapy from 3 to 7%. Therefore, sequential measurement of blood glucose is important for the early diagnosis of type 1 diabetes. It has been reported that the glucotoxicity effect on β-cells may result in ketoacidosis even in patients with type 2 diabetes. However, the possibility that type 2 diabetes existed in our subjects was considerably low for the following reasons: *1*) patients with ketosis-prone type 2 diabetes have a significantly greater rate of insulin discontinuation at 6 months after onset (25), *2*) all autoantibody-negative patients with the acute-onset form have absent β-cell function at 12 months after onset, and *3*) 14 patients who had been diagnosed as having type 2 diabetes many years before the diagnosis of type 1 diabetes were positive for anti-islet autoantibodies.

In conclusion, interferon treatment–related type 1 diabetes develops rapidly in the majority of patients, and stronger antiviral treatment with PegIFN and ribavirin may induce earlier development of type 1 diabetes. To avoid life-threatening events such as diabetic ketoacidosis, early detection of the development of type 1 diabetes by sequential monitoring of anti-islet autoantibodies and blood glucose before and during interferon therapy is important. Furthermore, patients who develop interferon treatment–related type 1 diabetes are genetically susceptible.

Acknowledgments—This study was supported by a grant-in-aid from the Japan Diabetes Society.

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

K. Nakamura and E.K. wrote the manuscript and researched data. A.I. and T.A. contributed to discussion and reviewed and edited the manuscript. H.I., Y.U., and T.K. reviewed and edited the manuscript. A.S. and K. Nakanishi contributed to discussion and reviewed and edited the manuscript. H.M. and T.M. reviewed and edited the manuscript. T.H. contributed to discussion and reviewed and edited the manuscript.

The authors thank T. Ichikawa, Division of Gastroenterology and Hepatology, Nagasaki University Hospital, Japan, and H. Yatsuhashi, Department of Hepatology, NHO Nagasaki Medical Center, Japan, for their helpful discussions. The authors acknowledge the editorial assistance of Gordon Murphy, KN International, Inc., Hoffman Estates, Illinois.

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総　説

統計ソフトRによるロジスティック回帰分析

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はじめに

統計ソフトR[1]はインターネットなどを通して配布されている無料の統計解析ソフトである。R言語によるプログラミングとしてのスクリプトを書いて，それを実行するという使い方をする。S-plusという商品の統計ソフトがあって，これはS言語によりスクリプトを記述するのであるが，2つの言語には高い互換性がある。歴史的にはソフトSが1975年頃から作られ始め、ソフトRは1993年頃からSとは独立にしかしS言語を採用して開発されてきているとのことである。現在RやSを対象とする書籍が英文，和文とも非常に多数出版されているので，今後Rは急速に普及するであろうと私は思っている。RjpWiki (http://www.okada.jp.org/RWiki/)には90冊のRの和書，またR総本山のサイト(http://www.r-project.org/)には114冊のRの洋書のリストがある。

RはPackagesの集合体である。ひとつのPackageには特定の統計解析手法のためにその著者がインプリメントした統計解析関数がまとめられている。Packageごとに著者は異なっている。Rのインストールでは，多くのPackagesに共通して必要となる基本部分や使用頻度が高い手法などが納められた30個ほどのPackagesだけがインストールされる。その他のPackagesは必要が生じたときにインターネットから追加でインストールして使用する。

今回私は，Kleinbaum, Kleinの本[2]にある2つのデータを用いて，Rによるロジスティック回帰分析を試みた。これはソフトには依らないロジスティック回帰分析の本であるが，巻末にSAS，SPSS，STATAでの扱いが書いてある。さて色々と調べると，Rにはロジスティック回帰分析を行うことのできるPackagesは複数存在していることが分かり，それらのいくつかを試用した。しかし，いずれも単独のPackageだけで私が得たい出力をすべて得るには不十分であった。最終的に私なりに，十分に満足のいく方法とは言えないものの，数個のPackagesを組み合わせる方法に落ち着いた。本稿ではこの私の試みを報告する。ロジスティック回帰分析のためのRの使い方の詳細やスクリプトはここには書かない。私が医学会で行った講演のスライド[3]で，分かり易いユーザー・インターフェースを持つ統計ソフトJMP[4]とRとを対比させて，ロジスティック回帰分析の計算法や計算結果を詳しく説明したので，スライドもご覧下さい。

1．ロジスティック回帰分析

ロジスティック回帰分析は臨床的なデータの解析において最もよく使われている統計解析法のひとつと思われる。目的変数或いは従属変数と言われるyは通常のロジスティック回帰分析では2値変数である。2値で表示される調査項目が目的変数yになる。2値それぞれが起きる確率Pが興味の対象であるという場合に，ロジスティック回帰分析が使われる。例えばyが冠動脈性心疾患の有/無である場合，興味は疾患の有病率Pである。1－Pはいわば無病率ということになる。患者に関するデータの中のその他の種々の項目はロジスティック回帰分析では説明変数と呼ばれる。コレステロール値，収縮期血圧，拡張期血圧などの数値変数と，喫煙歴の有/無(2値)，高血圧症の有無(2値)，年齢階級(例えば40代，50代，60代，70以上の4値)などの名義変数の区別がある。

ロジスティック回帰分析では，一人の被験者の目的変数や説明変数のデータ値から，

$$h=\beta_0+\beta_1x_1+\cdots+\beta_kx_k,\ P=1/(1+e^{-h})$$

という確率Pを表わすモデル回帰式を設定する。各β_ix_iをここでは回帰式の項と呼ぶ。項は一つの説明変数の項や，2つの説明変数の組み合わせの項(2次の交互作用項とよぶ)などで，複雑なモデルでは3つの変数の組み合わせの3次交互作用項などを考えることもある。確率Pに及ぼす影響の度合いが，項ごとにp値やオッズ比で示

される。ロジスティック回帰分析を行って論文を書くときは，各項のp値やオッズ比とその信頼区間を論文中に記載するのが普通である。それによって，冠動脈性心疾患の危険因子がひとつの説明変数やそれらの交互作用の中から選び出されることになる。目的変数yに対する危険因子を選び出すことが興味の対象である場合にも，ロジスティック回帰分析が使われる。

目的変数yが2値の場合を扱う通常のロジスティック回帰分析の一般化として，yが3値以上の場合を扱う方法がある。簡単のために3値で説明することにして，値を0，1，2と表す。yは名義変数で0，1，2は単に分類項目名である。0，1，2の間に0＜1＜2のように順序を設定する方法と順序を設定しない方法があり，前者をordinalロジスティック回帰分析，後者をpolytomousロジスティック回帰分析と呼ぶ。スライド[3]において私が行った解析を例として挙げれば，腫瘍の分化度が前者の解析の例，癌腫の種類(腺癌，扁平上皮癌，その他)が後者の例である。これらの目的変数が多値のロジスティック回帰分析が用いられる状況は上述の2値の場合と同様で，目的変数yの確率が興味の対象である場合や，yに対する危険因子を選び出すことが興味の対象である場合である。

2．ロジスティック回帰分析のモデル式

上にロジスティック回帰分析のモデル回帰式の形を示した。$x_1, x_2, \cdots, x_k$には一人の被験者のデータに基づく値が代入され，それらの係数$\beta_1, \beta_2, \cdots, \beta_k$は回帰係数と呼ばれる未知パラメータである。項$\beta_i x_i$に一つの連続説明変数を対応させる場合には，一人の被験者のこの連続変数値をx_iに代入する。項$\beta_i x_i$に一つの2値名義説明変数を対応させる場合には，統計ソフトRでは0または1がx_iに代入される。値0を代入する方の名義変数値をreference値と呼ぶ。一例として，変数が喫煙歴の有無の場合に，0に無しを1に有りを対応させればreference値は「無し」である。

さて，4値の名義説明変数がひとつあると，それは3つの項を消費してしまう。仮に4値名義変数を年齢階級とし値は40代，50代，60代，70以上の4個で40代をreference値として，3つの項($\beta_1 x_1, \beta_2 x_2, \beta_3 x_3$)を割り当てるとすると，40代，50代，60代，70以上のそれぞれにRでは(0,0,0)，(1,0,0)，(0,1,0)，(0,0,1)を(x_1, x_2, x_3)に代入する。3個の未知パラメーター($\beta_1, \beta_2, \beta_3$)を用いることによって，単に40，50，60，70という等間隔の数値とは違う扱いで，年齢階級の4値の影響を捉えることができる。

2次交互作用項の例として，陽性，陰性の2値の名義変数ECG（心電図結果）とHPT（高血圧症）の交互作用を考えよう。陽性は値1，陰性は値0とする。この場合交互作用項はβ×ECG×HPTという項で，βは未知係数でECG×HPTはそれぞれの変数値の積である。したがってECGとHPTが両方陽性のときECG×HPTは1，その他のとき0となる。このように一般に2次の交互作用項は2つの説明変数の積に未知パラメータを掛けたものである。2次の項β×ECG×HPTをモデルに含める場合には，必ずその構成要素のECGの項とHPTの項もモデルに含めるという規則をみたすhierarchically well-formulated model[5]だけを考えるのが普通である。ECGとHPTの両方が同時に陽性となった場合は，モデル式のhにはβ_{ECG}とHPTの係数β_{HPT}が加わる。しかし$\beta_{ECG}+\beta_{HPT}$よりさらに大きい影響が有病率に対して存在するか，または逆により少ない影響しかないかのいずれかの場合には，それを調整するために交互作用項が必要となるのである。

2値変数yの値が冠動脈性心疾患の有/無で，したがってPは有病率である場合，被験者のデータに基づいて$x_1, \cdots, x_k$の値をPのモデル回帰式に代入したときにこの人の有病率が得られるように，未知の回帰係数($\beta_0, \beta_1, \cdots, \beta_k$)の値を算出するのがロジスティック回帰分析である。

3．説明変数の選択

私が用いた冠動脈疾患の有/無を目的変数yとし，したがってこの疾患の有病率をPとする事例には，数値変数3個，2値名義変数4個，4値名義変数1個が用意されている。説明変数が8個なので，2次の交互作用項は28通り考えられるので，2次の交互作用まで考慮したモデルは36個の項のそれぞれをモデル式に含めるか否かを検討することになる。ただし先に述べたようにhierarchically well-formulated modelだけを考える。このようなモデル式の項の選択では，ステップワイズ法を使うのが一つの方法である。ステップワイズ法では項の採否の基準が必要で，統計ソフトJMPでは基準として，p値，AIC，BICの3つのどれかを選ぶことができるのであるが，Rではp値を基準にするステップワイズ法を行うPackageは見つけられなかった。ちなみに，或る解析を現在のバージョンのRでは行えないという結論を得ることは中々難しい。多数のPackagesに目を通すことが大変だからである。さて，私個人的にはp値を基準にするステップワイズ法が好みで，理由はこの方法によって選択されたモデルでは変数ごとのp値が全て＜0.05になるようにできるからである。しかしスライド[3]では，JMPとRとで共通に行えるAICを基準にしたステップワイズ法を行った。

JMPのステップワイズ法の欠点もついでに述べておこう。JMPのマニュアル[6]のp.130から引用すると,「従来のステップワイズ回帰では,モデルにカテゴリカルな項があるケースを想定していなかったため,名義尺度または順序尺度の項は,ダミー列として扱われます。水準が2つしかないときは列が1つしか生成されないため,ダミー列で行っても特に問題はありません。しかし,3水準以上ある場合は,複数の列を処理しなければなりません。」とある。年齢階級という4値変数(40代,50代,60代,70以上)には2節で述べたように3個のダミー変数(x_1, x_2, x_3)が割り当てられるのであるが,JMPの変数選択はx_iレベルの選択がなされ,年齢階級をひとまとまりとした選択がなされないのである。例えば変数増加法において,まずx_3が入り次に他の変数が入り次にx_2が選択されるというようなことが起き,3個の(x_1, x_2, x_3)全体の採否という扱いがなされない。x_3だけが入った後の他の変数の採否というのは,結果の解釈に苦しむだけである。3値以上のカテゴリカルな変数の存在を想定したステップワイズ法に早く改善して欲しい所である。

4. 最大尤度法

ロジスティック回帰分析に用いられるデータの中の被験者の人数をn人とする。説明変数の値を2節の様に代入したPをn人分用意して,yが疾患有りならPを,疾患無しなら1−Pを用いたn個のPや1−Pの積を尤度関数(likelihood function)といいLと表わす。未知ベクトル$\beta=(\beta_0,\beta_1,\cdots,\beta_k)$の関数である。Lが最大になるように未知ベクトルの値を決める方法を最大尤度法といい,求められた値を$(\beta_0,\beta_1,\cdots,\beta_k)$の最尤推定量という。線形重回帰分析の場合の回帰係数の値を決める原理として最小2乗法が良く知られているが,線形重回帰分析のyに正規分布を仮定した場合,最小2乗法と最大尤度法は一致する。ロジスティック回帰分析では最小2乗法は通常はもはや用いられない。

最大尤度法により導かれる未知ベクトル$\beta=(\beta_0,\beta_1,\cdots,\beta_k)$の最尤方程式は,線形重回帰分析では行列演算によって代数的に解くことが出来るが,ロジスティック回帰分析では代数的には解くことが出来ない。そこでニュートン法により,初期値$\beta^{(0)}$から初めて,順次$\beta^{(1)}$, $\beta^{(2)}$, …, $\beta^{(m)}$, …と近似解を求めてこれが収束したら極限値を最尤推定量とみなすという方法が用いられる。ニュートン法はソフトとして実現するのが難しい方法である。ロジスティック回帰分析においても,説明変数の個数が多くて交互作用も含むモデルにおいては最尤方程式が複雑になり,近似解の列が発散したり最大尤度解とは別の解に収束したりすることが起こる可能性がある。正しい解を得るために重要な点のひとつに,初期値$\beta^{(0)}$はできるだけ最大尤度解の近くに取るということがあり,統計ソフトは独自にこのための工夫をしている。ロジスティック回帰分析の統計ソフトの信頼性は,このニュートン法が適格であるかどうかにかかっている。2010年にMo[5]が行った9個のロジスティック回帰分析統計ソフトの信頼性の試験では,Rを含む6個のソフトは信頼性があるという結論が得られた。この論文では統計ソフトSASでニュートン法が発散することが度々起こったことが報告されている。私が今回の講演をするに当たって行った計算では,単独の説明変数の項5個(4値名義変数1個を含む)と交互作用項5個の,未知ベクトルβが17次元のモデルでは,ソフトRとJMPの計算結果は大きく食い違ってしまった[3]。少なくとも一方のソフトは,最大尤度解を求めることに失敗しているということである。ちなみに単独の説明変数の項5個(4値名義変数1個を含む)の未知ベクトルβが8次元のモデルでは,両方のソフトの結果は一致した。

5. ロジスティック回帰分析の診断

ロジスティック回帰分析が済んで回帰式を求めた後で,データは的確にロジスティックモデルに当てはまっているかどうか検討することを診断と呼ぶ。診断も詳しく行おうとすると,多数の事項を検討することになる。私はROC解析を試みた。データの目的変数(心疾患の有無)とロジスティック回帰分析による疾患の有無の鑑別から2×2クロス表を作り鑑別の感度,特異度を調べる解析である。ロジスティック回帰分析による鑑別とは,カットオフ値cを決めて,分析で得られた被験者の有病率Pに対してP≥cならこの被験者は疾患有りとすることである。ROC解析においてROC曲線を描いて得られる曲線の下の面積AUC (area under the curve)が,ロジスティック回帰分析の当てはまりの程度を表す目安となる。

現在のRにはROC解析を行うPackagesは多数存在するようである。私は2, 3個のPackagesを試すうちにROCR[7]が気に入ったので,それ以上に他のPackagesは試さないでROCRを使うことに決めた。ちなみに私は,Rを扱った本に説明してあるPackageを選ぶ以外に,ソフトに依らない統計学の本やSASやSPSSの本などからデータと解析結果の双方が書いてある例題を選んで例題を計算してみることでPackagesを選んでいる。

ROCRは2005年頃に発表されたようである。The R Book[8]は2004年に出版された本で,種々の統計解析を解析ごとに異なる著者が執筆した章からなる例題集である。ロジスティック回帰分析も収録されている。付属の

CDにすべての章のRスクリプトと解析に使われたデータが載っているのであるが，ロジスティック回帰分析の章だけ著作権の関係かデータが載っていない。私は以前にこの本のこの章をRの計算を行いながら読んだが，乱数などを交えて一通りの数値を埋めた後で，本の結果に似た結果が得られるように数字を調整したデータを自作して計算して，この本の解析の流れを体験した。The R Bookのロジスティック回帰分析の章で著者は分析の診断としてROC解析を行っているが，ROC解析やROC曲線描画のスクリプトは自作している。著者がインプリメントしたROC解析スクリプトもCDに収録されている。私はこの章を読んだときにRのROC解析のPackageを探したが，当時は良いPackageを見つけられなかった記憶がある。

6．私が選んだRのロジスティック回帰分析のPackages

回帰係数β_iについて$\beta_i=0$の仮説検定には，Wald検定と尤度比検定(likelihood ratio test)がある。Wald法は回帰係数($\beta_0, \beta_1, \cdots, \beta_k$)の最大尤度解が，データの例数を無限大にすると正規分布に収束するという定理に基づいて，β_iの分布がその推定値と標準誤差とをパラメータとする正規分布にしたがうとみなす方法である。小さい標本数でロジスティック回帰分析を行う場合には，Wald法は根拠のない方法となり望ましくないので，尤度比検定を使う方がよいとされている(Kleinbaum, Klein[2], p.139, JMP9 モデルおよび多変量[6], p.182)。

Rではロジスティック回帰分析は，glm関数によるのが定番のようである。これは統計学の中でgeneralized linear modelと言われる広範な分野を占める一連の解析手法を行うための関数で，ロジスティック回帰分析はその1項目である。glm関数はlm (linear model)関数と並んでRの代表的な関数のひとつで，Rの初期インストールに含まれる。

さて，glm関数がオッズ比の信頼区間を出力しないことが，まず私にとって問題となった。オッズ比の信頼区間は回帰係数β_iの信頼区間から直ちに得られるので，β_iの信頼区間の出力の問題と言い換えてもよい。もっともglm関数はβ_iの推定値と標準誤差を出力するので，1行のRスクリプトを書くだけでWald法に基づいたオッズ比の信頼区間が得られるので，glm関数の著者は「オッズ比の信頼区間も出力されますよ」というであろうと思う。私としてはユーザー・インターフェイスの観点から，直接にオッズ比の信頼区間という出力項目を持つRの関数を使うことにこだわった。それを探してepicalc[9] Packageを使うことに落ち着いた。epicalcはRのパッケージ・メニューからRに追加のインストールをする必要がある。epicalcのlogistic.display関数がglm関数の出力からWald法でオッズ比の信頼区間を計算する。また，Wald検定と尤度比検定のp値も出力する。

Packageの著者が書いたepicalcの解説を[9]を見るうち，ordinalやpolytomousのロジスティック回帰分析は，generalized linear modelの1項目であるにもかかわらずglm関数では計算できなくて，Rでは前者はMASS Packageのpolr関数，後者はnnet Packageのmultinom関数で計算することが分かった。ちなみにMASS Packageは，著者の本Modern Applied Statistics With S[10]からPackage名が取られている。これはRによる種々の統計解析の事例集であり日本語訳[11]も出版されているが，格調が高くて難しい本である。

次に，Rのepicalcはロジスティック回帰分析については2つの検定法をサポートしているが，ordinalやpolytomousのロジスティック回帰分析ではどちらの検定のp値も出力しないことが，私にとっての問題点となった。polr関数やmultinom関数がβ_iの推定値と標準誤差を出力するので，1行のRスクリプトを書くだけでWald検定がなされるのであるが，私はここでは特に尤度比検定の出力が欲しかった。

再びインターネットでRのPackageを検索して，car PackageのAnova関数がpolr関数やmultinom関数の出力を受けて尤度比検定を行うことがわかった。以上で私がスライド[3]で行った解析の関数は出揃った。

7．その後のこと

回帰係数β_iについて$\beta_i=0$の検定にはWald検定と尤度比検定があることを6節で述べたが，β_iの信頼区間の算出にもWald法とprofile likelihood法がある。オッズ比の信頼区間はβ_iの信頼区間から直ちに得られる。標本数が少ない場合のロジスティック回帰分析では，オッズ比の信頼区間はprofile likelihood法を用いることが望ましい。JMPでは目的変数が2値のロジスティック回帰分析では，profile likelihood法によるオッズ比の信頼区間が出力される。一方ordinalやpolytomousのロジスティック回帰分析ではJMP ver.9はオッズ比の信頼区間をまったく出力しない。

Rのepicalcは3種のロジスティック回帰分析のWald法のオッズ比の信頼区間を出力するという所までが，スライド[3]の内容であった。その後に分かったことであるが，6節で述べたMASS Packageのconfint関数がglm関数の出力を受けてprofile likelihood法によるオッズ比の信頼区間を，またconflict.default関数がWald法による信頼区間を出力する。実際に計算をした所，スライド[3]ではprofile likelihood法のJMPとWald法のRの結果はわ