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Relationships among 64k Autoantibodies, Pancreatic β-cell Function, HLA-DR Antigens and HLA-DQ Genes in Patients with Insulin-Dependent Diabetes Mellitus in Korea

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Objectives: *Among autoantibodies detected in patients with insulin-dependent diabetes mellitus(IDDM), antibodies to 64,000Mr islet protein(64k), now recognized as glutamic acid decarboxylase(GAD), appear to be an even more predictive marker of IDDM than islet cytoplasmic antibody(ICA) or insulin autoantibody(IAA). We examined the relationships among 64k autoantibodies, pancreatic β-cell function, HLA-DR antigens and HLA-DQ genes in patients with IDDM in Korea.*

Methods: *To identify the 64k autoantibody, the immunoprecipitation method was performed for 35 patients with IDDM and 10 normal controls. In patients with IDDM, serum C-peptide levels were measured and HLA-DR typings and HLA-DQA1 and DQB1 gene typings were performed.*

Results: *12 of 35(34%) patients with IDDM were positive for 64k autoantibody in contrast to none of 10(0%) normal controls. There were no differences in residual pancreatic β-cell function between 64k autoantibody positive and negative groups. 64k autoantibody was detected more frequently in patients with recent(duration<6 months, 10/25[40%]) and young-aged(age<15 years, 7/18[39%]) onset of IDDM. All of 3(100%) patients with HLA-DR3/DR4 heterotypes were positive in 64k autoantibody, in contrast to 1 of 7(14%) patients without HLA-DR3 nor DR4. The frequencies of HLA-DQA1*0301, HLA-DQB1*0201, DQB1*0302 and DQB1*0303 gene types were higher in patients with 64k autoantibody(12/12[100%]) vs. without 64k autoantibody 18/22[81%], 5/11[45%] vs. without 64k autoantibody 5/22[23%], 5/11[45%] vs. without 64k autoantibody 8/22[36%] and 6/11[55%] vs. without 64k autoantibody 9/22[41%].*

Conclusions: *There results suggest that 64k autoantibodies have some relationship with HLA-DR, DQA1 and DQB1 genes, but not with residual pancreatic β-cell function in Korean patients with IDDM.*

Key Words : *64k autoantibody, Insulin-dependent diabetes mellitus, Residual pancreatic β-cell function, HLA-DR, DQA1, DQB1, Korea*

INTRODUCTION

Insulin-dependent diabetes mellitus(IDDM) is caused by a chronic, clinically silent autoimmune process that gradually destroys the insulin-producing beta-cells. In the early pre-clinical phase of IDDM, antibodies to islet cell antigens are detected. Indeed, some IDDM-associated antibodies, which are present months to years before the onset of that metabolic disease, identify individuals who are high risk for IDDM. The ability to identify such individuals reliably may allow thera-

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peutic intervention with immunoregulatory agents to prevent IDDM[1,2].

The most practical markers of beta-cell autoimmunity currently available are circulating antibodies against islet antigens and they are islet cell cytoplasmic antibody(ICA)[3], islet cell surface antibody(ICSA)[4], insulin autoantibody(IAA)[5], and Mr-64000(64kD) autoantibody etc. Among these autoantibodies, an antibody to Mr-64kD antigen, which is a component of pancreatic beta-cells, was detected about 10 years ago[6]. Mr-64kD protein recently appears to be the gamma-aminobutyric acid(GABA)-synthesizing enzyme glutamic acid decarboxylase(GAD) and is regarded as a major target antigen in Type 1 diabetes[7]. 64k autoantibodies are detected at a high frequency in new onset Type 1 diabetic patients and may precede clinical onset by several years and they have also been detected in pre-diabetic period in the first degree relatives of Type 1 diabetic patients who later develop diabetes[8-10]. 64k autoantibodies may be detected when there are no residual pancreatic beta-cell function[11] but not associated with pancreatic β-cell function[10]. The prevalence of 64k autoantibody may be influenced by genetic background including HLA-DR, DQ types[8,12].

There is an unanswered question: why are there marked differences in the prevalence of GAD autoantibodies in Caucasoid Type 1 diabetic patients(63%) compared with various Asian populations(5-31%)(P. Zimmet, unpublished observations). In this study, we report the analysis of the relationships between the prevalence of the 64k autoantibodies, residual pancreatic β-cell function, HLA-DR antigens and HLA-DQ genes in Korean patients with IDDM.

SUBJECTS AND METHODS

1. Patients

The 35 unrelated patients with IDDM attending the Yonsei University Medical Center were selected with these entry criteria: the occurrence of ketoacidosis at onset or a history of being ketosis-prone, body mass index(BMI) less than 25kg/m^2, fasting plasma C-peptide level less than 0.6ng/ml and being replaced with dailyinsulin injection to control blood glucose concentrations due to absolute insulinopenia[13,14]. Some patients, whose initial fasting plasma C-peptide levels were higher than that of diagnostic criteria(0.6ng /ml), but their fasting C-peptide levels were markedly lower absolutely, thereafter, or there were episodes of being ketosis-prone within follow-up duration, were included in this study. Control sera(n=10) were obtained from healthy individuals without diabetes or a family history of diabetes(mean age 24 years, range 19-31). The clinical characteristics of IDDM patients and normal controls are listed in Table 1.

2. Methods

Beta-cell function was assessed, after an overnight fasting and postprandial 2 hours, by measuring plasma C-peptide levels using the radioimmunoassay method(RIA kit, Incstar Co., USA). The plasma C-peptide levels, measured at the time of first attending our hospital, were selected and samplings were obtained for the study of 64k autoantibody.

For 64k antigen determination, rat pancreatic islets were isolated from fresh rat pancreases. Islet cells were metabolically labelled with ^{35}S-methionine and immunoprecipitated with test sera and antibody bound proteins were electrophoresed through 10% sodium dodecylsulphate polyacrylamide separating gels. We confirmed the 64kD band by the immunoprecipitation method(Fig. 1).

Serologic typings of the HLA-DR antigens were determined by the lymphocyte microcytotoxicity method(One Lambda Co., USA). HLA-DQAl genotypes for 34 diabetic patients were done by the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method and HLA-DQB1 genotypes for 33 patients were done by the PCR-RFLP and PCR-sequence specific oligonucleotide method.

Student's t-tests were used to compare the mean of each data and the Chi square test to determine the statistical signif icance between

Table 1. Clinical Characteristics of IDDM Patients and Normal Controls

	IDDM patients	Normal controls
No	35	10
Sex(M/F)	13/22	4/6
Age(yr)	19±10	24±6
range	4-49	19-31
Onset of age(yr)	14±10	-
range	2-46	
Duration of diabetes(yr)	1.7±3.3	-
range	0.02-17	
64k Ab positivity(%)	12/35(34)	0/10(0)

group frequencies. $p<0.05$ was considered statistically significant.

RESULTS

The mean age of the 35 IDDM patients was 14 years(range 4-49years) and the mean duration of diabetes was 1.7years(range 0.02-17years). The 64k autoantibody was detected in 12 of 35(35%) patients with IDDM and in none of 10(0%) normal controls(Table 1).

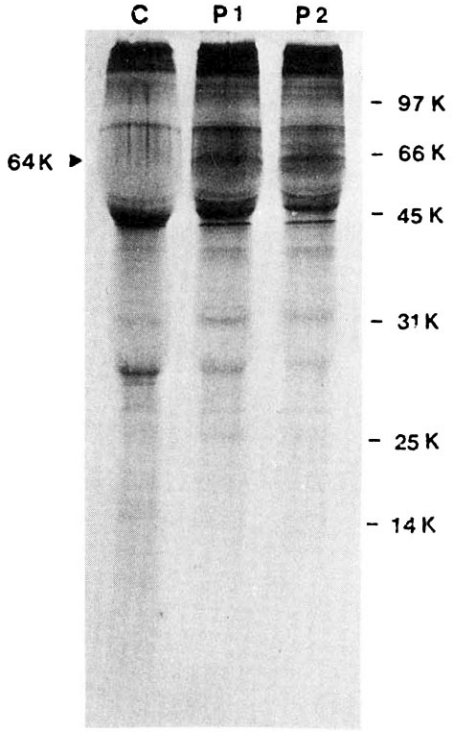

Fig. 1. 64k band confirmed by immunoprecipitation method.

1. Clinical Characteristics and Pancreatic Beta-cell Function

According to the duration of diabetes in IDDM patients, 64k autoantibody was detected in 10 of 25(40%) patients with a duration of diabetes of less than 6 months and none of 3 patients with a duration between 6 months and 2 years, and 2 of 7(29%) patients with a duration longer than 2 years(Table 2). According to the onset-age of diabetes in IDDM patients, 64k autoantibody was detected in 7 of 18(39%) patients with onset-age less than 15 years and 5 of 15(33%) patients with onset-age between 16 and 35years, and none of 2 patients with onset-age older than 36years(Table 3).

In 25 patients whose duration of diabetes was less than 6 months, there were no significant dif

Table 2. 64k Autoantibody Prevalence According to the Duration of Diabetes in IDDM Patients

Duration	No	%
<6m	10/25	40
6m-2yr	0/ 3	0
2yr<	2/ 7	29
Total	12/35	34

Table 3. 64k Autoantibody Prevalence According to the Age of Onset in IDDM Patients

Age of onset(yr)	No	%
<15	7/18	39
16-35	5/15	33
36<	0/ 2	0
Total	12/35	34

Table 4. Clinical Characteristics of New-onset IDDM Patients with or without 64k Autoantibody(N=25, D<6m)

	64k Ab Positive	64k Ab Negative	Significance
No	10	15	—
Sex(M/F)	2/8	9/6	—
Age(yr)	17±9	19±12	NS
range	4-31	4-49	
Onset age(yr)	12±8	15±12	NS
range	2-26	2-46	
Duration(m)	2.0±1.3	2.3±1.5	NS
range	0.2-4	0.4-6	
C-peptide(ng/ml)			
fasting	0.81±0.56	0.87±0.48	NS
PC2h	1.45±0.91	1.12±0.74	NS

values : mean±SD, NS : no significance

Table 5. Clinical Characteristics of All IDDM Patients with or without 64k Autoantibody(N=35)

	64k Ab Positive	64k Ab Negative	Significance
No	12	23	—
Sex(M/F)	3/9	10/13	—
Age(yr)	19±9	20±11	NS
range	4-31	4-49	
Onset age(yr)	12±8	15±10	NS
range	2-26	2-46	
Duration(yr)	2.1±5.1	1.5±2.1	NS
range	0.02-17	0.1-8	
C-peptide(ng/ml)			
fasting	0.73±0.55	0.83±0.47	NS
PC2h	1.27±0.91	1.08±0.74	NS

values : mean±SD, NS : no significance

Table 6. HLA-DR Antigen Typing in IDDM Patients(N=34)

	No	%
$DR3^+$	8/34	24
$DR4^+$	22/34	65
$DR9^+$	12/34	35
$DR3^+/DR4^+$	3/34	9
$DR3^+/DR9^+$	2/34	6
$DR4^+/DR9^+$	4/34	12
$DR3^+$ or $DR4^+$	27/34	79
$DR3^-/DR4^-$	7/34	21
$DR3^+$ or $DR4^+$ or $DR9^+$	33/34	97
DRX/DRX	1/34	3

X : HLA-DR antigens other than DR3 or DR4 or DR9

Table 7. Prevalence of 64k Autoantibody According to the HLA-DR Antigens in IDDM Patients(N=34)

	No	%
$DR3^+/DR4^-$	2/ 5	40
$DR3^-/DR4^+$	5/19	26
$DR3^+/DR4^+$	3/ 3	100
$DR3^+$ or $DR4^+$	10/27	37
$DR3^-/DR4^-$	1/ 7	14

Table 8. HLA-DQA1 Gene Typing in IDDM Patients(N=34)

	No	%
$DQA1^*0301^+$	30/34	88
$DQA1^*0401^+$, $^*0501^+$	17/34	50
$DQA1^*0301^+/DQA1^*0401^+$, $^*0501^+$	13/34	38
$DQA1^*0301^+$ or $DQA1^*0401^+$, $^*0501^+$	34/34	100
$DQA1^*0301^-/DQA1^*0401^+$, $^*0501^+$	0/34	0

Table 9. Prevalence of 64k Autoantibody According to the HLA-DQA1 Genes in IDDM Patients

DQA1	Total	64k Ab Positive	64k Ab Negative
	No(%)	No(%)	No(%)
*01#	1/34 (3)	—	1/22 (5)
*0101	5/34(15)	1/12 (8)	4/22(18)
*0102	3/34 (9)	1/12 (8)	2/22 (9)
*0103	3/34 (9)	—	3/22(14)
*0301	30/34(88)	12/12(100)	18/22(81)
*0401, *0501	17/34(50)	6/12 (50)	11/22(50)

ferences in fasting and postprandial 2 hours plasma C-peptide levels between 64k autoantibody positive and negative groups. Similarly, in all 35 patients, regardless of the duration of diabetes, there were no differences between the two groups(Table 4, 5).

2. HLA-DR Antigens and 64k Autoantibody

Among 34 patients with IDDM, the number of patients who had DR3, DR4 and DR9 were 8(24%), 22(65%) and 12(35%) respectively, and the number of patients who had either DR3, DR4, or DR9 was 33(97%)(Table 6). All 3(100%) patients with HLA-DR3/DR4 heterotype were positive in 64k autoantibody and 10 of 27(37%) patients with either HLA-DR3 or DR4 were positive, and 1 of 7(14%) patients without DR3 nor DR4 was positive in 64k autoantibody(Table 7).

3. HLA-DQA1 and DQB1 Gene Types and 64k Autoantibody

Among 34 patients with IDDM, the number of patients who had HLA-$DQA1^*0301$ and $DQA1^*0401$, ^{*0501} were 30(88%) and 17(50%) respectively and all 34 patients had either $DQA1^*0301$ or $DQA1^*0401$, ^{*0501}(Table 8). The frequency of HLA-$DQA1^*0301$ was higher in patients with 64K autoantibody(12/12[100%] vs. without

Table 10. HLA-DQB1 Gene Typing in IDDM Patients (N=33)

	No	%
DQB1*0201+	10/33	30
DQB1*0302+	14/33	42
DQB1*0303+	14/33	42
DQB1*0201+ or DQB1*0302+ or DQB1*0303+	27/33	81
DQB1*X/DQB1*X	6/33	19

X : HLA-DQ genes other than DQB1*0201 or DQB1 *0302 or DQB1*0303

Table 11. Prevalence of 64k Autoantibody According to the HLA-DQB1 Genes in IDDM Patients

DQA1	Total	64k Ab Positive	64k Ab Negative
	No(%)	No(%)	No(%)
*0201	10/33(30)	5/11(45)	5/22(23)
*0301	2/33 (6)	—	2/22 (9)
*0302	13/33(39)	5/11(45)	8/22(36)
*0303	15/33(45)	6/11(55)	9/22(41)
*0401	11/33(33)	3/11(27)	8/22(36)
*0402	1/33 (3)	—	1/22 (5)
*0501	3/33 (9)	1/11 (9)	2/22 (9)
*0601	1/33 (3)	—	1/22 (5)
*0602	1/33 (3)	—	1/22 (5)
*0603	1/33 (3)	—	1/22 (5)
*0604	4/33(14)	—	4/22(18)
*0605	1/33 (3)	1/11 (9)	—

64k autoantibody 18/22[81%])(Table 9).

Among 33 patients with IDDM, determined by their HLA-DQB1 gene typings, the number of patients who had DQB1*0201, DQB1*0302, DQB1*0303 and DQB1*0401 were 10(30%), 14(42%), 14(42%) and 11(33%) respectively, and 27 patients(81%) had either of them(Table 10). The frequencies of HLA-DQB1*0201, DQB1*0302 and DQB1*0303 were higher in patients with 64k autoantibody(5/11[45%] vs. without 64k autoantibody 5/22[23%], 5/11[45%] vs. without 64k autoantibody 8/22[36%] and 6/11[55%] vs. without 64k autoantibody 9/22[41%])(Table 10, 11).

DISCUSSION

The 64k autoantibody assay, developed by Baekkeskov et al[6] in 1982, involves the immunoprecipitation of radiolabled islet membrane fractions by patient sera and now revealed GAD[7]. There are two GAD isoforms, Mr-65,300 (GAD_{65}) and Mr-66,660(GAD_{67}), which are highly expressed in the brains of higher vertebrates and rat pancreatic islet cells[15], but also expressed in testes, ovaries, adrenal medulla etc. There are no known biochemical differences in GAD between brain and pancreatic islets and isolated human pancreatic islets demonstrated an exclusive expression of the GAD_{64} encoding transcription in contrast to rat pancreatic islets, and brains of vertebrates expressed both GAD isoforms[16,17].

The role and significance of islet GAD and GABA are unclear, these are expressed only in beta-cells of the pancreas[18], regardless of insulin secretory granules, which reside in cytoplasm[19,20]. The GABA produced by GAD appears to be involved in the regulation of glucagon secretion in the islets and may have other physiological roles as well[21]. In isolated pancreatic islet-cell cultures, synthesis of GAD was increased according to glucose concentration[22,23] and GAD-associated antibodies have been described as having an inhibitory effect on islet insulin secretion[24]. These findings emphasize the potential role of GAD in the pathogenesis of IDDM and make it important to characterize its biological function in the islets and to elucidate its potential pathogenic involvement in beta-cell destruction. Although 64k autoantibody may have greater sensitivity, specificity and predictive value for human IDDM, than other established markers[10,11,25-27], the 64k autoantibody assays have not been widely used because islet-based immunoprecipitation assays are expensive and laborious, preventing completion of large studies[28-32].

Among the autoantibodies of IDDM, the best studied is ICA, and several groups have reported that ICA is a good predictive marker in relatives of patients with IDDM[33-36]. High and persistent titers for ICA, especially in the pediatric age-group, are reliable predictors[36]. It was known that ICA was detected about 80% sera of patients with recent-onset IDDM, but usually appears after onset of diabetes and a rapid drop in serum titer during disease progression, compared to 64k autoantibody[37,38]. The presence of ICA may indicate the prognostic marker of IDDM, rather than a pathogenic role to develop diabetes. The Mr 64000(64k) islet cell protein, now recognized an glutamic acid decarboxylase (GAD), is a major target antigen in Type 1 diabetes. Immunoprecipitating antibodies to this protein have been found in 80% of newly diagnosed cases[1,37], and they have also been detected in the pre-diabetic period in first degree relatives of Type 1 diabetic patients who later develop dia-

betes[38,39]. 64k autoantibody may persist, even when there are no residual beta-cell functions, but is rarely detected in the normal population[16,37-40]. Zimmer et al[12] reported that there were marked differences in the prevalence of GAD antibodies in Caucasoid Type 1 diabetic patients (63%) compared with various Asian populations (5-31%).

In our study, 64k autoantibody was detected in 12 of 35(34%) patients with IDDM but in none of 10(0%) normal controls. Among patients studied, there was a patient whose duration of diabetes was 17 years, the fasting plasma C-peptide level was less than 0.1ng/ml, was positive in 64k autoantibody. According to these results, 64k autoantibody seems not to have sensitivity but has specificity to be a diagnostic marker of IDDM in Korea, and 64k autoantibody may persist even though there is no residual pancreatic β-cell function. Hagopian et al[11] reported that there was a tendency that the prevalence of 64k autoantibodies are increased in patients with recent-onset diabetes and younger-aged patients. In our study, 64k autoantibody was detected in 40% of patients of recent onset(duration< 6months), and in 39% of patients of the younger-aged group(age<15years) in contrast to 34% of overall patients with IDDM. Several groups[38,41] reported that, at the time IDDM developed, ICA positive patients lost their pancreatic beta-cell function more rapidly than ICA negative patients. In the high risk group of diabetes, like first relatives of patients with IDDM, the presence of 64k autoantibody is a better predictive marker of a decrease in their insulin secreting functions and becoming diabetic patients than other autoantibodies[7-9]. But Tuomi et al[10] reported that there were no differences in pancreatic beta-cell function between 64k autoantibody positive and negative groups. In our study, there were also no differences in fasting and postprandial 2 hours plasma C-peptide levels of recent-onset(duration <6momths) and overall patients between 64k autoantibody positive and negative groups. According to these results, the presence of 64k autoantibody may not be a predictive marker of residual pancreatic beta-cell function and insulin secretory capacity. But ICA positive IDDM patients have nearly the same insulin secretory function as ICA negative patients during their first 3 months of diabetes and, thereafter, they lost their secretory function more rapidly[10]. To confirm the exact relationship between 64k autoantibody and pancreatic β-cell function, further and serial studies are warranted.

In Caucasians, 50-60% of the population have HLA-DR3 or DR4, which are known to be a genetic susceptibility factor of IDDM, and over 95 % of patients with IDDM have at least one of them[25,42]. But a smaller percent population of Asians, including Koreans, have less HLA-DR3 or DR4 than Caucasians and this difference may explain why there are fewer IDDM patients in Asians[12,27,42,43]. Seiβler et al[9] reported that there was an increased prevalence of 64k autoantibody significantly in normal non-diabetic children who have HLA-DR4 and HLA-DQ β chain with 57th non-asparic acid, than those without any of them. In the report of Serjeantson et al[12], among Australian patients with IDDM, heterozygous for HLA-DR3 and DR4, 85% were positive for antibodies to GAD, significantly different from the prevalence of 48% in patients with, at least, one HLA-DR antigen other than DR3 or DR4, and these observations may reflect differential genetic and environmental interactions in IDDM or differential persistence of GAD antibodies in those with different genetic backgrounds. In Caucasians, HLA-DR3 and DR4 confer a particular risk alleles for susceptibility to IDDM[42], whereas in the Chinese, the high risk are HLA-DR3 and DR9[4]. In the Japanese, in whom HLA-DR3 is virtually absent, the high risk IDDM alleles are HLA-DR4 and DR9[45]. In our study, among patients, heterozygous for HLA-DR3 and DR4, all 3 patients were positive to 64k autoantibody and 10 of 27(37%) patients, with at least one of them, were positive, but only 1 of 7(14%) patients without DR3 nor DR4 was positive to 64k autoantibody. Accordingly, HLA-DR3 and DR4, especially HLA-DR3/DR4 heterozygote, may be associated with the presence of 64k autoantibody.

Recently, HLA-DQ gene analysis is becoming more meaningful for the predictive marker of genetic susceptibility of IDDM, rather than HLA-DR antigens[1], and there were several reports that individuals with HLA-DQA1*0301 and HLA-DQB1*0302 were significantly more susceptible to IDDM than those without them[1,46,47]. According to studies about Koreans, Lee et al[48] reported that HLA-DQA1*0301, DQB1*0201 and DQB1*0303 genes were significantly increased in patients with IDDM than normal controls, and Hong et al[49] and Hahn et al[50] reported that HLA-DQA1*0301 gene was significantly increased in pa-

tients with Korean IDDM patients than the normal population. But there is no report about the relationship between HLA-DQ genes and the prevalence of 64k autoantibody in Korea. In our study, among 34 patients, the number of patients who have HLA-DQA1*0301 and DQA1*0401, *0501 are 30(88%) and 17(50%) respectively, and all 34 patients had at least one of them. All 12 patients who were positive to 64k autoantibody have DQA1*0301 in contrast to 18 of 22(82%) patients negative to 64k autoantibody. In the HLA-DQB1 gene analysis, the patients with HLA -DQB1*0201, DQB1*0301 and DQB1*0303 genes showed slightly increased prevalences, respectively, in 64k autoantibody positivity than those without them.

In our study, a small number of patients and a smaller prevalence of 64k autoantibody, compared to Caucacians, had limited statistically significant results. Further serial and large scale studies of 64k autoantibody are warranted to understand the pathogenesis of IDDM and the exact role of that autoantibody.

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