Original Article

Genetic characteristics and β-cell Autoimmunity in T1DM Children

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Summary:

Background: TIDM is known to be polygenic disease that appears from the interaction of mutation in multiple genes including HLA. The autoimmune mediated destruction of pancreatic β-cells is reflected by the presence of autoantibodies against prominent antigens in the pancreatic β-cells.

Objective: This study was designed to investigate the role of HLA-class I and class II antigens in the etiology of type 1 diabetes mellitus (T1DM) and also assessment of glutamic acid decarboxylase (GAD_{65}) autoantibodies in the patients at the onset of the disease.

Patients & Methods: Sixty T1DM patients who were newly onset of the disease (diagnosed less than five months) were selected. Eighty apparently healthy control subjects, matched with age, sex and ethnic backgrounds underwent the HLA-typing by lymphocytotoxicity assay. Finally 50 healthy individuals were selected randomly to undergo serological assessment of GAD_{65} autoantibodies using IRMA method.

Results & Conclusion: At HLA-class I region, T1DM patients showed a significant increased frequency of antigen A9 (40.0 vs.18.75%) and B8 (28.33 vs.8.75%) as compared to control subject. At HLA-class II region, DR3 and DR4 were significantly increased in patients (53.33 vs.26.25% and 50.0 vs. 12.5% respectively) as compared to controls. In addition to that, T1DM was significantly associated with DQ2 (33.33 vs.15%) and DQ3 (40.0 vs. 20%) antigens as compared to controls, suggesting that these haplotypes had a role in disease susceptibility, while the frequency of DR2 and DQ1 antigens were significantly lowered in patients compared to controls (6.66 vs. 25% and 6.66 vs. 22.5% respectively). These molecules might had protective effect.

Anti-GAD_{65} autoantibodies were present in 50% of T1DM children especially in older ages and in females more than males. High proportion of GADA was found in the patients carrying HLA-DR3/DR4 heterozygous. In conclusion, susceptibility to T1DM is genetically controlled.

Key words: T1DM patients, HLA, GAD_{65} autoantibodies.

Introduction:

Type 1 Diabetes Mellitus (T1DM) characterized by an autoimmune process that results in the destruction of insulin- producing β- cells, leading to insulin deficiency. It occurs worldwide and can appear at any age, from birth to the age around 30 years (1).

The disease generally shows a peak for clinical onset between 10-14 years of age with a sharp drop in late teens (2). The pubertal peak in onset of T1DM occurs early in girls than boys (3), since the hormonal changes of puberty differ between the sexes. Genes regulated by sex hormones could play an important role in the different patterns of the disease presentation. The gene for IL-6 is a possible candidate as its promoter is regulated by 17- β estradiol (E2) (4).

Genomic studies have confirmed that the main locus defining the genetic susceptibility to T1DM is encoded within the Major Histocompatibility Complex, HLA (Human Leukocyte Antigen) region on human chromosome 6 (5). The role of HLA alleles in T1DM was first indicated by the association with HLA-B8, -B15, and -B18 (6), and then

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with HLA-DR3 and DR4 encoded by the DRB1 locus and susceptibility with the DQB1 and DQA1 genes, which are in linkage disequilibrium with DR3 and DR4 (7,8,9). Another studies showed that A1, B8, and DR3 were the high risk antigens (10), while A24, B8, B15, DR3, DR4, DQ2, and DQ3 were highly associated with T1DM among Iraqi patients (11). Strong natural protection against T1DM is also conferred by the DR2.DQ6 haplotypes (12), which occurs in approximately 20% of the healthy white population but it is rarely found among patients with diabetes. DR2, DQ1, DQ4, and B35 were found among the protective alleles in Iraqi patients (10, 11).

The Autoimmune mediated destruction of pancreatic β- cells is reflected by the presence of autoantibodies against prominent antigens in the pancreatic β-cells. The HLA type of the individual may control the recognition of certain autoantigens including insulin, glutamic acid decarboxylase (GAD_{65} and GAD_{67}), membrane proteins that are homologous to tyrosin phosphatase (ICA512 and IA-2), and islet neuroendocrine ganglioside (13). The frequency of GAD autoantibodies (GADA) has been reported to vary from 0.5 – 3% among children from background population (14), from 6.4 - 13% among siblings of children with T1DM (15), and from 62 - 84% among patients with newly diagnosed disease (16). GADA has been reported to be associated with the DQ2 alleles (17), and DR3.DQ2 haplotypes (18). During normal immune responses, molecules encoded by DR&DQ genes bind and present peptide fragments of protein antigens to lymphocytes of the CD_4 subset. These class II molecules could play a pivotal role in the development of T1DM through presentation of islet-cell specific peptides to autoimmune CD_4 T lymphocyte (19). Cytotoxic T-cells recognizing an HLA-A2- restricted peptide of GAD have been found in prediabetics and patients with recent onset disease, but not in normal controls, although increased disease risk associated with HLA-A2 has not been found (20).

In Iraqi patients, we have no available data on the occurrence of T1DM in relation to genetic predisposition and presence of GAD_{65} autoantibodies on the age at onset of the disease. In the present study, we investigated the role of genes and humoral immune responses of diabetic patients through the HLA polymorphism and GAD_{65} autoantibodies.

Subjects, Materials and Methods:

Subjects:

Sixty Iraqi Type 1 diabetic patients (28 males and 32 females) were subjected to this study. The patients were attending to National Diabetes Center at Al-Mustansiriya University during the period May 2004 to October 2005. Their ages range from 3 -17 years, and they were new onset of the disease (diagnosis was from one week up to five months). For the diagnosis of Diabetes Mellitus, the criteria as listed in the Expert Committee of Diagnosis and Classification of Diabetes Mellitus, (2003) was used. All the patients were treated with daily replacement doses of insulin at the time of blood sampling. For the purpose of comparisons, 80 apparently healthy control subjects matched for age (4-17 years old), sex and ethnic back ground (Iraqi Arabs) were selected who have no family history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group for HLA typing. Out of these 80 controls, 50 healthy subjects (25 males and 25 females) were randomly selected for assessment of GAD_{65} autoantibodies, and for this test the patients were divided into two groups according to their ages: 36 child equal or less than 10 years and 24 child more than 10 years.

Collection of Blood Samples:

Ten milliliter of venous blood was collected from each patient as well controls. Eight milliliter of blood was put in heparinised test tube (10 U/ml) used for lymphocyte separation for the detection of HLA polymorphism, heparinised blood was processed as soon as possible. The remaining blood was collected into plain test tubes, then the serum was separated by centrifugation at 2500 rpm for 10 min., and kept at-20°C for the assessment of GAD_{65} autoantibodies.

Serological Typing of HLA Antigen:

Microlymphocytotoxicity assay is a complement dependent reaction based on the reaction of HLA anti- sera (Biotest, Germany) which recognize the correspondent membrane

bound antigen on the viable human lymphocytes in the presence of rabbit's complement (Biotest, Germany).

The Peripheral Blood Lymphocytes (PBLs) were isolated from the whole blood using Ficoll- isopaque gradient centrifugation (Flow-Laboratories,UK) originally described by Boyum, (1968) that was reported by Schendel *et al.,* (1997). This test was carried out in the Histocompatibility Laboratory in Al-Karama Hospital. The lymphocyte population were separated by nylon wool method to T-cells used in the phenotyping of HLA- class I (A, B, and C) antigens and B- cells used for phenotyping of class II (DR and DQ) antigens (23). Cells were counted and determined their viability. The viability accepted should be 95% and above. The final cells accepted was adjusted to $2\text{-}3x10^6$ cells /ml. Microlymphocytotoxicity assay was used for both HLA- class I and class II typing (24).

Assessment of serum anti- GAD_{65} autoantibodies:

Serum GADAs was measured by Immunoradiometric assay (IRMA) using anti-GAD IRMA kit (Immunotech Beckman Coulter, France).

Statistical analysis:

Regarding of HLA and disease association the frequency distribution for selected variables was done first. The strength of disease association with particular HLA antigen was determined by calculating the relative risk (RR) and etiological fraction (EF), and if the association is negative, therefore the preventive fraction (PF) was calculated (25). The significance of such association was assessed by Fisher exact probability. Chi square tests were used for the measurements of correlation and dependency among other variable observations.

Results:

HLA Antigen Association

The distribution of HLA-A; -B; -C; -DR; and -DQ antigens with their frequencies in T1DM patients and controls are presented in table (1), while antigens showing significant variations between patients and controls are given in table (2).

At HLA-A locus, the antigen A9 was significantly increased (P= 0.004) in the patients and such differences were associated with RR value of 2.88 and EF value of 0.261. This positive association remained significant after correction (PC= 0.032).

At HLA-B locus three antigens were significantly increased (B8, B12, and B15) in the T1DM patients (P=0.002, 0.032, and 0.018 respectively) in comparison to controls. The frequency of these antigens were (28.33 vs. 8.75%; 11.66 vs. 2.50%; and 11.66 vs. 2.00% respectively) and such differences associated with RR values of (4.122, 5.150, and 9.113 respectively) and EF values of (0.214, 0.093, and 0.103 respectively). However one positive association remained significant after correction (PC= 0.032) and this was with B8 while both B12 and B15 return to non significancy (PC= 0.512 and 0.288 respectively). In contrast, the B35 and B51 antigens significantly decreased in the patients compared to controls (3.33 vs. 13.75% and 15.0 vs. 28.75% respectively), but such negative association also failed to remain at a significant level after correction (PC=0.496 and 0.656 respectively).

At HLA-C locus, Cw7 antigen significantly increased in T1DM patients (31.66 vs. 16.25%, P=0.026, RR=2.388, and EF= 0.183). Such positive association was felt after correction (PC= 0.104). In the other hand, the Cw4 antigen significantly decreased in the patients than in controls (6.66 vs. 18.75%, P=0.031), but the negative association failed again to retain a significant level after correction (PC=0.124).

Table 1: HLA antigen frequencies in controls and T1DM patients

HLA-antigens	T1DM patients (Number = 60)		Controls (Number =80)	
HLA-A locus	**No.**	**%**	**No.**	**%**
A1	12	20.00	15	18.75
A2	26	43.33	30	37.50
A3	5	8.33	7	8.75
A9	24	40.00	15	18.75
A10	12	20.00	10	12.50
A11	0	ND	16	20.00
A19	16	26.66	32	40.00
A28	7	11.66	8	10.00
HLA-B locus				
B7	6	10.00	6	7.50
B8	17	28.33	7	8.75
B12	7	11.66	2	2.50
B13	2	3.33	2	2.50
B14	0	ND	4	5.00
B15	7	11.66	1	2.00
B16	6	10.00	2	2.50
B17	0	ND	1	1.25
B18	2	3.33	4	5.00
B27	2	3.33	5	6.25
B35	2	3.33	11	13.75
B37	7	11.66	4	5.00
B40	3	5.00	2	2.50
B41	5	8.33	8	10.00
B51	9	15.00	23	28.75
B73	2	3.33	2	2.50
HLA-C_W locus				
Cw**2**	3	5.00	3	3.75
Cw4	4	6.66	15	18.75
Cw5	2	3.33	2	2.50
Cw7	19	31.66	19	16.25
HLA-DR locus	**No.**	**%**	**No.**	**%**
DR1	21	35.00	18	22.50
DR2	4	6.66	20	25.00
DR3	32	53.33	21	26.25
DR4	30	50.00	10	12.50
DR5	2	3.33	1	11.25
DR6	6	10.00	3	3.75
DR7	14	23.33	12	15.00
DR8	11	18.33	8	10.00
DR10	4	6.66	0	ND
HLA-DQ locus				
DQ1	4	6.66	18	22.50
DQ2	20	33.33	12	15.00
DQ3	24	40.00	16	20.00

Table 2: Antigens of HLA-class I and class II regions showing significant variations between T1DM patients and controls.

HLA	TIDM vs. control				
	RR	EF	PF	P	PC
A2	–	–	–	–	–
A9	2.88	0.261		0.004	0.032
B8	4.122	0.214	–	0.002	0.032
B12	5.150	0.093	–	0.032	NS
B15	9.113	0.103	–	0.018	NS
B35	0.216	–	0.107	0.031	NS
B51	0.437	–	0.162	0.041	NS
Cw4	0.309	–	0.128	0.031	NS
Cw7	2.388	0.183	–	0.026	NS
DR2	0.214	–	0.195	0.003	0.027
DR3	3.210	0.366	–	$9.7x10^{-3}$	0.008
DR4	7.00	0.428	–	$1x10^{-5}$	$9x10^{-5}$
DR5	–	–	–	–	–
DQ1	0.246	–	0.168	0.008	0.024
DQ2	2.833	0.215	–	0.009	0.027
DQ3	2.666	0.249	–	0.008	0.024

RR: relative risk; EF: Etiological fraction; PF: Preventive fraction; P: Fisher exact probability; PC: Corrected probability

At HLA-class II region (DR-loci), three antigens showed different frequencies in patients and controls, these were DR2, DR3, and DR4. Increased frequencies of DR3 (53.33 vs. 26.25%) and of DR4 (50.0 vs. 12.5%) were observed in patients. The positive association RR values were of 3.210 and 7.00 respectively and EF values of 0.366 and 0.428 respectively. Such positive association was highly significant ($P=9.7x10^{-3}$ and $1x10^{-5}$ respectively) and remained highly significant after correction (PC=0.008and 9x10-5 respectively). In contrast DR2 antigen significantly decreased in the patients (6.66 vs. 25.0%). Such negative association was significant (P=0.003) and remain significant after correction (PC=0.027).

At HLA-DQ loci, two antigens DQ2 and DQ3 were significantly increased in the patients compared with controls (33.33 vs. 15.0%, P=0.009, RR=2.833, EF=0.215) for DQ2 while (40.0 vs. 20.0%, P=0.008, RR=2.666 and EF=0.249) for DQ3. This positive association remained significant after correction (PC=0.027and 0.024 respectively). The antigen DQ1 was significantly decreased in T1DM patients (6.66%) vs. (22.5%) in controls, such negative association (P=0.008) remained significant after correction (PC=0.024).

GAD_{65} autoantibodies:

GADA were detected in 30 of Iraqi children with newly diagnosed T1DM (50%). The proportion of index cases positive for the both age groups in comparison with controls were shown in table (3). A higher significant proportion of the patients was positive to GADA in both age groups (18/36, 50% and 12/24, 50% respectively) as compared to control groups (1/21; 4.76% and 2/29; 6.9% respectively). This differences were highly significant $P_1=0.0001$.

A higher significant proportion of the girls tested positive for GADA (5/9;

55.6%) were observed in age group >10 years old than of girls ≤10 years old (11/23; 47.8%), (P_2= 0.049), table (4), while the proportion of boys tested positive for GADA was higher in age group ≤10 years old than >10 years old (7/13; 53.8% vs. 7/15; 46.7%), but this difference fails to be significant (P_2= 0.804). No statistical differences were observed between males and females in each age group (P_1= 0.729 and 0.673 respectively).

Table 3: Differences of sero positive / negative of GADA between control and T1DM patient groups.

Age	Groups	No.	Sero positive		Sero negative		P_1	P_2
			No.	%	No.	%		
≤10 years	Controls	21	1	4.76	20	95.24	Chi 0.0001 (HS)	Chi 1.00 (NS)
	T1DM	36	18	50.00	18	50.00		
>10 years	Controls	29	2	6.90	27	93.10	Chi 0.0001 (HS)	
	T1DM	24	12	50.00	12	50.00		

P_1 : Patients vs. controls P_2 : Patients ≤10 years vs. patients >10 years.

NS: Not significant

Table 4:Differences of sero positive / negative of GADA between control and T1DM males and females patients.

Parameter	≤10 years (n=36)					>10 years (n=24)					
	GADA+		GADA–		P_1	GADA+		GADA–		P_1	P_2
	No.	%	No.	%		No.	%	No.	%		
Males	7	53.8	6	46.1	Chi 0.729 (NS)	7	46.7	8	53.3	Chi 0.673 (NS)	Chi 0.804 (NS)
Females	11	47.8	12	52.2		5	55.6	4	44.4		Chi 0.049 (S)

P_1: males vs. females P_2 : patients ≤10 years vs. patients >10 years.

NS: Not significant

Relation of HLA-DR, -DQ Risky Alleles with Sero-Positive GADA in T1DM Patients:

Table (5) represented the distribution of sero-positive GADA in patients with HLA-DR risky alleles, and in those with other different alleles.

The proportion of sero-positive GADA in patients with HLA-DR risky alleles were significantly higher (P= 0.001) than those who had other alleles. The DR3/DR4 combination seemed to have the high prevalence (53.33%) compared to DR4 (10.0%) and DR3 (6.67%).

Table 5: Distribution of sero-positive GADA in T1DM patients and relation with HLA-DR risky alleles.

Parameter	No.	DR3/DR4 No. (%)	DR3 No. (%)	DR4 No. (%)	Others No. (%)	P
GADA +ve	30	16 (53.33)	2 (6.67)	3 (10.0)	9 (30.0)	0.001 (S)

Chi = 16.523

The results represented in table (6) indicate a high proportion of $GADA^+$ in patients carrying DQ3 risky allele (43.33%) compared to DQ2/DQ3 (23.33%) and DQ_2 (10%). By using chi-square test, the statistical analysis showed a significant differences of sero-positive GADA in patients with DQ risky alleles than those carrying other alleles (P= 0.016).

Table 6: Distribution of sero-positive GADA in T1DM patients and relation with HLA-DQ risky alleles.

Parameter	No.	DQ2/DQ3 No. (%)	DQ3 No. (%)	DQ2 No. (%)	Others No. (%)	P
GADA +ve	30	7 (23.33)	13 (43.33)	3 (10.00)	7 (23.33)	0.016 (S)

Chi = 5.05

Discussions:

HLA Association Alleles:

It is known that T1DM has been transferred from prediabetic subjects to an HLA identical sibling as a consequence of bone marrow transplantation (26). The present study detected that immunogenetic predisposition may be considered as an important factor for the development of T1DM in association with the HLA antigens in which

markers of human HLA showed different distributions in patients and controls.

At HLA class I region, significant increased frequencies of antigen A9 and B8 were observed in the patients. Other HLA T1DM association studies carried out in other world population revealed an association with other HLA-class I antigens, B15 in Canadian population (6), B8 and B15 in Finnish population (27), in addition to A1, A2, B56, B62, Cw3 and Cw7 (28), A24 in Japanese (29). Such differences can be explained in the ground of racial differences, especially if we consider that HLA antigens show different frequencies in different populations including Iraqis. HLA-A1 and B8 were found to be associated with T1DM in Basrah population (10). Al-Samarrai, (2001) found a very high significant association of HLA-A24, B8 and B15 with T1DM in her study conducted in Baghdad**.** Other positive association were observed in the tested T1DM patients (B12, B15 and Cw7) but the significance was lost when the probability was corrected for the number of antigens tested at each locus and such statistical application is important to exclude a chance occurrence of an association due to many comparisons that were made (30).

At HLA-class II region, further antigens had positive associations with T1DM. These were DR3, DR4, DQ2 and DQ3. The polymorphism of HLA-class II loci has gained much interest in the HLA disease association studies. However, multiple studies have reported association between HLA-DR and DQ phenotypes and T1DM. DQ2.DR3 and DQ3.DR4 haplotypes reported as high risk alleles in Caucasians (31), DR4, DQ4 but not DR3 were found to be dominant in Japanese (32), while DR3, DR4, DR9 and DQ2 were found the only alleles positively associated with T1DM in Koreans (33). In Finland, DQ2/DQ3 genotype was found to be associated with genetic susceptibility and was more frequent in children diagnosed <5 years of age (34), and in diabetes-associated autoantibodies emerged in children with predisposing HLA-DQ alleles after 3 months of age (35). In Lebanese 77% and 40% of T1DM patients were positive for DQ2 and DQ3 respectively (36). High significant association of HLA-DR3, DR4, DQ2 and DQ3 with T1DM was reported in Iraqi patients (11).

Studies of HLA genes at the molecular levels showed that this association with HLA-DR is secondary to a stronger link with certain HLA-DQ variants. The critical factor is the amino acid at position 57 in the HLA-DQB chain. Genetic variants of DQB which encode the amino acid aspartate at this position seem to confer protection against T1DM, whereas variants encoding other amino acids increase the risk. Hence the HLA-DR3 and DR4 association arises because these DR-alleles are linked to DQB alleles which do not encode aspartate (37). It is worthy to note that amino acid 57 in HLA-DQB lies in the "antigen binding groove". It was reported that class I HLA-24 gene promotes pancreatic β-cells destruction in an additive manner in the patients with T1DM susceptible HLA-class II genes (38). Antigens B35, B51, Cw4, DR2 and DQ1 showed a negative association with the disease, but after correction only the DR2 and DQ1 antigens remain significant. These antigens may have protective effect especially if we consider PF values to be 0.195 for DR2 and 0.163 for DQ1 antigens.

Clearly, the structural differences seen between the predisposing and protective HLA molecules will affect their ability to bind or interact with diabetogenic antigens and the T-cell receptor (TCR) of autoreactive β-cell specific T-cells (39). Several mechanisms have been proposed to explain how this might influence the risk of developing autoimmune T1DM: Protective HLA molecules may form stable complexes with self antigens in the thymus, leading to efficient deletion of potentially autoreactive T cells (negative selection). In contrast, the less stable complexes formed by the predisposing HLA-molecules may result in inefficient T-cell removal and the release of autoreactive T cells into the periphery (40). Predisposing and protective HLA molecules may interact differently with the TCRs of autoreactive T cells, affecting the phenotype of the T cells (proinflammatory versus regulatory) (39) or their activation status (Proliferative versus anergised) (41). This immunomodulatory hypothesis is supported by the observation that DQ1 can protect against the development of diabetes, even after the onset of β-cell autoimmunity (42).

GAD_{65} autoantibodies:

Islet cells reactivity as judged by the presence of antibodies to the GAD_{65} were

observed in 50% of the patients studied in both age groups (table 3). The present results indicated that older children were more often tested positive than younger ones in females (55.6 vs. 47.8%). This difference seems to be not significant between males in both age groups (table 4). This result is in disagreement with (17), but in agreement with (43) which indicated that GADA was less affected by age at clinical onset in patients than other autoantibodies marker. Our observation is in consistent with other studies and supports the notion that autoimmunity is more common among females more than 10 years old (17, 43).

The functional role of GADA in the pathogenesis of T1DM comes from their relationship to T-cell reactivity to GAD_{65} autoantigen. Presentation of an immunodominant T-cell epitope from the human GAD_{65} autoantigen is enhanced by GAD_{65} autoantibodies through increasing the efficiency of antigen capture by APCs including Fc receptor (FcR)-Positive monocytes/macrophages (19).

Relation of GADA with HLA:

The results in table (5) indicated that GADA were found at the highest levels in index cases carrying DR3/DR4 heterozygous. This indicates that GADA expression is regulated genetically. It is known that there is an over-representation of DR3/DR4 heterozygous subjects among young children with newly diagnosed T1DM as compared with adolescents and adults with recent-onset disease (44). These observations support the concept that a strong genetic susceptibility is associated with aggressive rapidly progressing β-cell destruction as reflected by marked GADA responses and clinical manifestation of T1DM at young age, while a weaker genetic predisposition results in a slower destructive process and disease presentation in adults. In this study a low frequency of GADA is observed in the patients who were homozygous for DR3. In contrast, other reports indicated that increased GADA concentration was the charactistic of DR3/DQ2 haplotypes (45, 17). Another study reported that only T-cell reactive with GAD-derived peptides in the context of DR-heterodimers could be isolated form the periphery of T1DM patients (46), indicating that HLA-DR rather than DQ seems to be the principle restriction element used by T-cells present at the onset of the disease.

Conclusions:

The HLA-class I (A9 and B8) and class II (-DR3, DR4, DQ2 and DQ3) antigens were significantly increased in T1DM patients and they played an important role in the etiology of the disease, while DR2 and DQ1 antigens were significantly decreased in the patients. GADA were present in 50% of diabetic children. Older children were tested positive for GADA more than younger ones, especially females and high proportion of GADA was found in the T1DM patients carrying HLA-DR3/DR4 heterozygous.

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