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ORIGINAL ARTICLE

Role of Human Leucocyte Antigen (HLA) Class II Gene Polymorphism in Type 1 Diabetes Mellitus

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RT-PCR,
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ABSTRACT

Background*: Type 1 diabetes (T1D) is a multifactorial disease caused by a complex interaction of genetic and environmental factors, which makes identification of disease causing variants very difficult. Knowledge of the genetics of T1D would allow better disease definition and improved ability to identify individual's risks.* ***Objectives****: Identification of the Human Leukocyte Antigen gene polymorphism in T1D Mellitus and its relationship to maternal and paternal gene polymorphism.* ***Methodology****: This study included 20 children (with their parents) with Type 1 Diabetes Mellitus who had regular follow up in the Paediatric Endocrinology Outpatient's Clinic Children Hospital, Minia University; during the period from June 2014 to March 2015. The studied groups were subjected to the followings; History taking, general and systemic examination and Real time PCR for detection of (DQB1* 0102, DQB1* 0302, DRB1*04) for the parents and the studied diabetic children.* ***Results****: Thorough history taking, general and systemic examination and RT- PCR for detection of (DQB1* 0102, DQB1* 0302, DRB1*04) for the parents and the studied diabetic children. Ten (50%) of the studied group had DQB1*0102 Gene versus 12 (60%) of their fathers and 11 (55%) of their mothers. Concerning DQB1*0302 Gene, 15 (75%) of the diabetic patients had it versus16 (80%) of their fathers and 15 (75%) of their mothers. Lastly, as regard DRB1*04 Gene 14 (70%) of the diabetic patients were positive versus 13 (65%) of their fathers and 11 (55%) of their mothers. There is a great importance of avoiding exposure to the different predisposing factors which may precipitate early onset of diabetes.* ***Conclusion:*** *The genetic makeup could determines the age of onset of type 1 diabetes. There is an importance of avoiding exposure to the different predisposing factors which may precipitate early onset of diabetes, this may help to delay it especially if it is genetically determined.*

INTRODUCTION

Type I diabetes (T1D) is the third prevalent chronic disease of childhood, affecting up to 0.4% of children. It is believed that large proportion of cases of type 1 diabetes result from the autoimmune destruction of the pancreatic ß cells, leading to complete dependence on exogenous insulin.[1] Knowledge of the genetics of T1D in our community would allow better disease definition and improved the ability to identify risky diabetic individuals and associated disorders.[2]

According to the results obtained, a formula, depending on the alleles detected, might be developed for prediction of the occurrence of the disease and more ambitiously, the approximate age of onset, if possible.

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This would be the first step towards T1D prevention.[2] The aim of our study was to identify the Human Leukocyte Antigen gene polymorphism in Type 1 Diabetes Mellitus and its relationship to maternal and paternal gene polymorphism.

METHDOLOGY

1. Study Population:

This study included 20 children (with their parents) with Type 1 Diabetes Mellitus who had regular follow up in the pediatric Endocrinology Outpatient's Clinic, Children Hospital, Minia University during the period from June 2014 to March 2015. Inclusion criteria of the patients group included; children of either sex were included, clear level of consciousness at time of the interview, cooperation to participate in the study while the exclusion criteria were; refusal to do investigations or high level of uncooperativeness of the patient or his parents, presence of chronic illness other than diabetes mellitus. A written informed consents were taken from

all patients' parents for approval of the entry in the study after explaining the study aim and procedures to them

a. History and physical examination

The studied groups were subjected to the followings: thorough History taking (demographic data and data related to parents), general including anthropometric data and systemic examination.

b. Samples collection

Four ml of blood samples were obtained from the diabetic patients in the morning after an overnight fasting of 8 hours and before insulin administration; blood was withdrawn by venipuncture after complete aseptic technique with Alcohol 70%.

c. Measuring of glucose and hemoglobin level

These were divided into 1 blood was used to measure glucose and assayed by using fully automated clinical chemistry auto-analyzer system Kb on elab 20i (Thermo Electron Incorporation Finland). One ml of blood was put in tube with EDTA for detection of HbA1c% by using resin column chromatography. Kit content was supplied by TECO DIAGNOSTICS, California, USA (Nathan et al, 1984)

2. DNA extraction

Blood samples were collected. Centrifuged serum samples were subjected to extraction. The extraction was done by automated Qiacube instrument (Qiagen extraction kit, U.S.A)

Extracted DNA was subjected to Real time PCR to detect different alleles (DQB1*0102 allele and DRB1*04 and DQB1*0302) which are common in T1D patients using suitable primers and SYBR Green and other Real time PCR. USB® VeriQuest® SYBR® Green qPCR Master Mix (2X) with Fluorescein Product number 75665. USB VeriQuest SYBR Green qPCR Master Mix with Fluorescein is a ready-to-use master mix optimized for SYBR Green detection on real-time PCR instruments that use fluorescein to perform dynamic well factor collection for data normalization (e.g. the iCycler iQTM, iQ5, or MyiQTM real-time PCR systems). The 2X master mix contains chemically-modified VeriQuest Taq DNA Polymerase, $MgCl_2$, ultrapure nucleotides with an optimized dUTP: dTTP ratio, Uracil-DNA Glycosylase (UDG), SYBR Green, and fluorescein in a proprietary reaction buffer. The hot start Taq DNA polymerase has no polymerase activity prior to the initial heat activation step which allows reaction assembly at room temperature as well as higher specificity and sensitivity. Since the mix contains dUTP and UDG, carry over contamination prevention can be performed prior to amplification. To generate a standard curve, a four-dilution standard was used. The real-time PCR instrument was operated according the thermal profile in the manual Ref (OD-0002-02). Each sample was spun down briefly in order to collect the Master Mix in the bottom of the reaction tubes, then the following protocol was performed as shown in table 1.

Table 1: Summarized procedure of PCR amplification

	UDG treatment	**Taq DNA polymerase activation and UDG inactivation**	**PCR amplification**	
	Hold	**Hold**	**30-45 Cycles**	
			Denature	**Anneal/Extend**
Temperature	50° C	95 ° C	95° C	60 °C
Time	2 minutes	10 minutes	15 seconds	30-60 seconds
Notes	Optional for carryover contamination prevention			Acquire real-time fluorescence data during this step

3. Statistical method

The collected data were coded, tabulated, and statistically analyzed using SPSS program (Statistical Package for Social Sciences) software version 20. Descriptive statistics were done for numerical data by mean, standard deviation and minimum& maximum of the range, while they were done for categorical data by number and percentage.

Analyses were done for parametric quantitative variables using one way ANOVA test for comparison between three groups and post Hoc Tukey's correction between each two groups. Analyses were done for quantitative variables using independent sample t test for parametric data between the two groups. Fisher exact test was used for qualitative data between groups. The level of significance was taken at (P value ≤ 0)

RESULTS

Table 2: Demographic data of the diabetic patients and their statistical analysis

Demographic Data		**Descriptive statistic**
Age (year)		
	Range	(2-18)
	Mean ± SD	11.12 ± 4.35
Gender		
Male	**No (%)**	8 (40%)
Female	**No (%)**	12 (60%)
Residence		
Urban	**No (%)**	3 (15%)
Rural	**No (%**)	17 (85%)
Age of onset (year)		
	Range	(1.8-17)
	Mean ± SD	7.54 ± 4.18
Duration of DM		
< 6 months	**No (%)**	7 (35%)
6-12 months	**No (%)**	0 (0%)
1-5 years	**No (%)**	8 (40%)
> 5 years	**No (%**)	5 (25%)
Socioeconomic status		
Ave	**No (%)**	15(75%)
Low	**No (%)**	5(25%)
History of consanguinity		
Positive	**No (%)**	10 (50%)
Negative	**No (%)**	10 (50%)
Family history of Type 2 DM		
Positive	**No (%)**	10 (50%)
Negative	**No (%)**	10 (50%)
Family history of Type 1 DM		
Positive	**No (%)**	1 (5%)
Negative	**No (%)**	19 (95%)

This study included 20 children (Table 2), their ages at the time of the study ranged from 2-18 years with a mean of 11.12 ± 4.35, the age of onset of DM was 1.8-17 years, with a mean of 7.54 ± 4.18. Twelve of them (60 %) were females and eight patients (40%) were males. Seventeen cases (85%) were from rural areas versus three (15%) from urban areas. Concerning the socioeconomic state, (75%) of them had moderate socioeconomic state and5 patients (25%) had low socioeconomic state. Ten families (50%) had consanguineous marriage (2nd degree and further) and the rest (50%) had non consanguineous marriage. One case (5%) had positive family history of T 1 D who was the father of the patient, while the rest (95%) had negative family history of T1D.

Regarding genetic results; table 3 and figures 1, 2 and 3 showed that 14 (70%) of the diabetic patients were positive for DRB1*04 gene versus 13 (65%) of their fathers and 11 (55%) of their mothers. Moreover, 10 (50%) of the studied group had DQB1*0102 gene versus 12 (60%) of their fathers and 11 (55%) of their mothers. Concerning DQB1*0302 gene, 15 (75%) of the diabetic patient had it versus 16 (80%) of their fathers and 15 (75%) of their mothers.

Table 4 showed that DQB1*0102 gene was negative in all family members in 4 cases (20%). While become positive in all family members in 7 cases (35%), also become positive in infant only in one case (5%), this gene was present in one parent only in 5 (25%) of cases and in one case (5%) this gene is represented in both parents only. Finally this gene was present in infant and at least one parent in 2 cases (10%). As regard DQB1*0302 gene, it was positive in all family members in 10 cases (50%), also it was positive in infant only in one case (5%), this gene was present in one parent in 3 (15%) of cases, in two cases (10%) this gene was represented in both parents. Finally this gene was present in infant and at least one parent in 4 cases (20%). Concerning DRB1*04 gene, it was not present at all family members in 3 cases (15%). While become positive in all family members in 8 cases (40%), also became positive in infants in two case (10%), this gene was presented in one parent in 2 (10%) of cases, in one case (5%) this gene was represented in both parents, finally this gene was present in infant and at least one parent in 4 cases (20%). Table 5 showed insignificant relationship between different genes positivity and some demographic data (P>0.05).

Table 3. Frequency of the studied genes in the diabetic group and their parents

	DRB1*04		**DQB1*0102**		**DQB1* 0302**	
	Positive No (%)	**Negative No (%)**	**Positive No (%)**	**Negative No (%)**	**Positive No (%)**	**Negative No (%)**
Diabetic patient	14 (70%)	6 (30%)	10 (50%)	10 (50%)	15 (75%)	5 (25%)
Father	13 (65%)	7 (35%)	12 (60%)	8 (40%)	16 (80%)	4 (20%)
Mother	11 (55%)	9 (45%)	11 (55%)	9 (45%)	15 (75%)	5 (25%)

Table 4. Description of each gene in each family member

	Descriptive statistics
	No (%)
DQB1*0102	
Negative in all	4 (20%)
Positive in all	7 (35%)
In infant only	1 (5%)
In one parent only	5 (25%)
In both parent only	1 (5%)
In infant and at least one parent	2 (10%)
DQB1 *0302	
Negative in all	0 (0%)
Positive in all	10 (50%)
In infant only	1 (5%)
In one parent only	3 (15%)
In both parent only	2 (10%)
In infant and at least one parent	4 (20%)
DRB1*04	
Negative in all	3 (15%)
Positive in all	8 (40%)
In infant only	2 (10%)
In one parent only	2 (10%)
In both parent only	1(5%)
In infant and at least one parent	4 (20%)

Table 5. Relationship between different genes positivity and some demographic data

	QB1*0102 (n=10)	**DRB 1*04 (n=14)**	**DQB 1 *0302 (n=15)**	**P value**		
				I *vs* II	**I *vs* III**	**II *vs* III**
Age of onset (years) Mean±SD	8.08 ± 4.11	7.5 ± 3.54	7.95 ± 4.24	0.995	0.999	0.989
Sex						
Male No (%)	4 (40%)	7 (50%)	7 (46.7%)	0.697	1	1
Female No (%)	6 (60%)	7 (50%)	8 (53.3%)			
1st presentation						
-DKA **No (%)**	5 (50%)	8 (57.1%)	11 (73.3%)	1	0.585	0.567
-Classical sym **No (%)**	3 (30%)	3 (21.45%)	3 (20%)			
-Accidentally discovered **No (%)**	2 (20%)	3 (21.45%)	1 (6.7%)			
Family history of DM						
-Ve **No (%)**	4 (40%)	6 (42.9%)	9 (60%)	1	0.428	0.356
+Ve **No (%)**	6 (60%)	8 (57.1%)	6 (40%)			
History of consanguinity						
-Ve **No (%)**	4 (40%)	9 (64.3%)	7 (46.7%)	0.408	1	0.340
+Ve **No (%)**	6 (60%)	5 (35.7%)	8 (53.3%)			
Residence						
Urban **No (%)**	1 (10%)	2 (14.3%)	2 (13.3%)	1	1	1
Rural **No (%)**	9 (90%)	12 (85.7%)	13 (86.7%)			

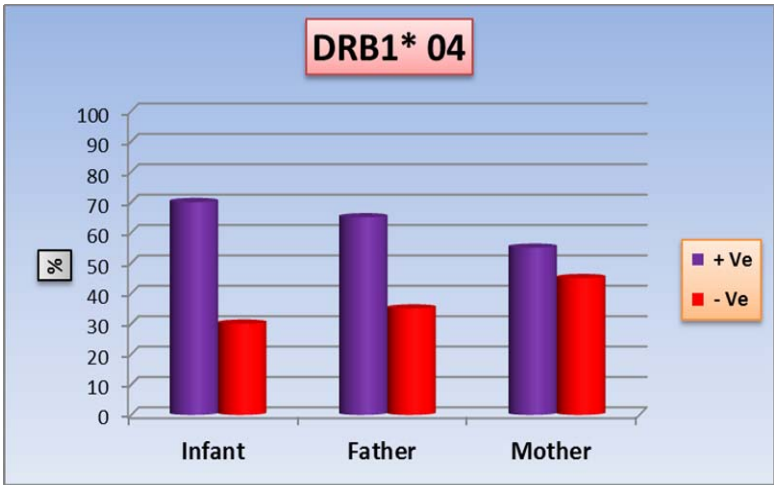

Figure 1. Frequency of DRB1*04 gene among diabetic patients and their parents

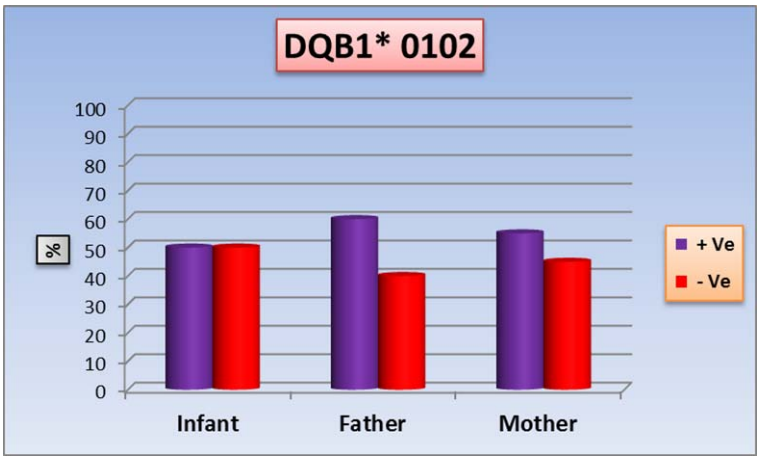

Figure 2. Frequency of DQB1*0102 gene among diabetic patients and their parents

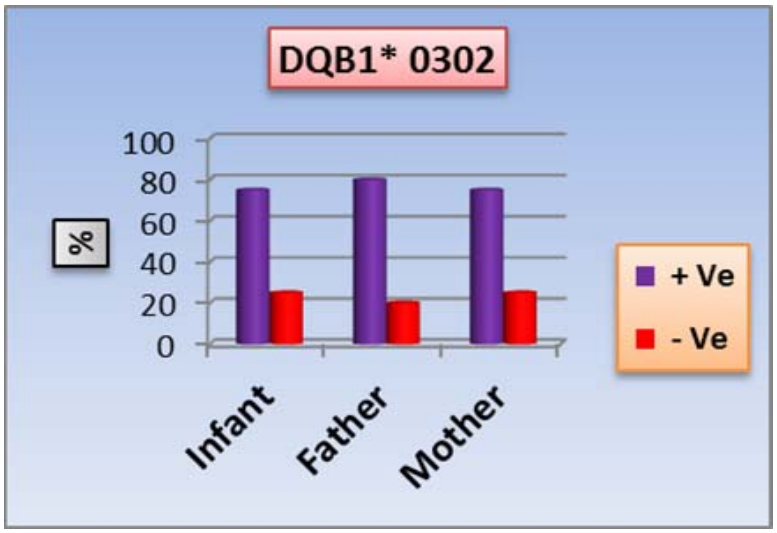

Figure 3. Frequency of DQB1*0302 gene among diabetic patients and their parents

DISCUSSION

It is known that Type 1 diabetes is a multifactorial disease caused by a complex interaction of genetic and environmental factors, with the former consisting of multiple susceptibility genes, which makes identification of disease-causing variants very difficult.[3] Therefore we aimed in this study to identify the Human Leukocyte Antigen (HLA) gene polymorphism in T1D and its relationship to maternal and paternal gene polymorphism. As regard the results of this study, the current study showed female predominance in 12 cases (60%) versus 8 (40%) were males. This result was in agreement with that previously reported.[4] In contrast, Soltesz et al[5] found that females and males were equally affected with T1D in young population while Alemu et al[6] reported a male predominance. Female predominance could be explained by that steroid hormones are considered the most likely factors that trigger the onset of female-gender-stratified, genetically-based autoimmune diseases.[7] That idea was supported by the increased prevalence of autoimmune diseases in women, the sexual dimorphism of the immune response and the *in vitro* modulatory effects of sex steroids on immune functions. These modifiers could directly or indirectly target steroid receptor that act as transcription factors for the susceptibility genes associated with T1D, although no such regulatory role for sex hormones has been identified.[7] However, this possibility is not without precedence, as sex hormones may act as critical modulatory factors that can induce disease expression.[8] Concerning residence, most of the studied cases 17 (85%) lived in rural areas, while 3 (15%) of them lived in urban areas. This result comes in agreement with the results reported by du Prel JB et al[9] that the risk of T1D is higher in children living in socially deprived areas.

Regarding family history of T1D, our study reported only one case (5%) with a positive family history of T1D who was the father of the patient, while the rest (95%) had negative family history. Our result was approximately in agreement with Warram et al[10] who found that the risk of a child developing T1D is about 10 % if the father has it. Moreover, several studies showed that there was a significant association between the family history of T1D and the disease.[11]

Identification of genes predisposing to T1D is important in establishing effective method for disease prediction, prevention and intervention.[12] There is a strong genetic variants to T1D pathogenesis. These include human leukocyte antigen (HLA) locus, in particular the class II region (DR and DQ), which accounts for 40- 50% of T1D familial clustering and non –HLA susceptibility loci, several of which were mapped by genome- scanning and/or candidate gene approach. There were studies in Egypt performed on genetic background of T1D which had mainly addressed the contribution of the HLA region as recently reported.[13]

The current study aimed to identify three main genetic loci reported to be involved in susceptibility to type 1diabetes in patients, their families, in order to determine the relative frequency of the different alleles.

According to our results it is evident that DQB1*0302 was the most frequent gene (75%), followed by DRB1*04 (70%) and DQB1*0102 (50%). This result was in agreement with Thomson et al[14] who stated that class II HLA, DRB1*04 and DQB1*0302, DQB1*0102 have been reported to be associated with T1D in almost all ethenic groups. Furthermore, the DR4 (DRB1*04 and DQB1*0302) haplotypes are positively associated with T1D.[11] This result also was in agreement with Stayoussef et al[15] who investigated the association of HLA DRB1 alleles among Arab population of Tunisia and Bahrain. Moreover, this finding was in agreement with Al-Hakbany M[16] who had a study on Saudi patients and found that higher risk was conferred by genotype DQB1*0302.

Our work revealed that 60% of those with positive DQB1*0102, 35.7 % of those with DRB1*04 and 53.3% of those with DQB1*0302 had positive consanguinity. This could be explained by the repetition of genotypes including HLA haplotypes, in the highly positive consanguinity rate.

CONCLUSION

Alleles or genetic variants associated with type 1 diabetes provide either susceptibility to or protection from the disease within a given environmental background. The genetic make up with the balance between susceptibility and protection alleles determines the age of onset of type 1 diabetes. In conclusion, It is important to avoid the exposure to the different predisposing factors which may precipitate to the early onset of diabetes especially if it is genetically determined.

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Ethics:

The study protocol was approved by the Council of Faculty of Medicine and its Institutional Review Board Each subject consented before participation in the study. The study protocol was approved by the Ethical Committee of Minia Faculty of Medicine. Written informed consent was obtained formal. The study protocol was approved by the Ethical Committee of Minia Faculty of Medicine. Written informed consent was obtained from all participants prior to participation in the study. The study was conducted in accordance

with the ethical guidelines of the 1975 Declaration of Helsinki.

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