Molecular and Demographic study about Type 1 diabetes mellitus and associated with HLA typing Class II in sample of Iraqi children

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Abstract

Background: Type1 diabetes mellitus is a serious disorder characterized destruction of pancreatic B-cell culminating in absolute insulin deficiency, the genetic factors contribute to the susceptibility of type 1 diabetes mellitus.

Objective: This study is carried out to increase knowledge demographic characters with type1 diabetes mellitus in children and its associated with Human Leukocyte Antigen (HLA) typing Class II

Subjects and methods: Fifty type1 diabetes mellitus patients compared to thirty apparently healthy individual were enrolled in this study with age ranged (3-13) years. Some demographic characters were analyzed and HLA were analyzed by polymerase chain reaction and sequence technique.

Result: This present study revealed that T1DM was found in 27 female and 23 male with ratio 1.2:1with out significant difference. It was noticed that the peak of occurrence of T1DM was at the age group 7-10 years, and onset age of T1DM 5-10 years with highly significant ($p<0.001$). There were 12(24%) of patients have positive family history, The second part of this study conducted on HLA typing Class II: DQA, DQB genes using two pairs of primers to amplify fragments with product size of 170bp and 270bp., for these genes respectively. The sequencing of PCR product for samples of patients showed many genetic variants such as DQA*0503, DQB*02,*03, for Class II compared with control.

Conclusion: The present study demonstrate that no significant difference between age and gender groups of T1DM patients, the highly incidence of T1DM occurrence was at the age group 7-10 years, it seems that association between the HLA Class II revealed by sequencing was finding the variation in DQA*0503,DQB*02,DQB*03 alleles.

Keywords: *DM diabetes mellitus. T1DM type1 diabetes mellitus. HLA Human Leukocyte Antigen.*

INTRODUCTION

Diabetes Mellitus (DM) is considered a major health issue, and describes a group of common metabolic and pathophysiologically disorders characterized by hyperglycemia. Within islets of Langerhans, the endocrine powerhouse of the pancreas are the insulin-producing pancreatic beta-cells, loss of beta-cell's mass and its function by inflammation and apoptosis is a major contributing factor to diabetes (Zammit etal., 2014). Importantly, the estimated prevalence of diabetes among adults in 2010 was 285 million (6.4%) worldwide, and it is suggested that this value is predicted to rise to about 439 million (7.7%) by 2030 (Shaw et al., 2010). According to American Diabetes

Association (ADA, 2014). Diabetes can be classified into four general categories which were Type1 diabetes mellitus (T1DM), Type2 diabetes mellitus (T2DM), Gestational diabetes (GDM) and specific type of disease due to other causes as disease.

Type1 diabetes mellitus is one of the most widespread chronic diseases of childhood and the most common type of diabetes in people under 40 year old, it is important cause of blindness, amputation, and end stage renal disease. The occurrence of T1DM has increased in many countries, whereas TIDM is the most common of diabetes, its specific etiology is not yet known. Its frequency varies in different racial and ethnic subgroups and is often associated with a strong familial, likely genetic, predisposition. (Jana et al., 2021)

The data suggest that genetic factors are involved with the development of the disease. Recently, there is evidence that more than 20 regions of the genome may be involved in genetic susceptibility to T1DM. However, none of the candidates identified have a greater influence on T1DM risk than that conferred by genes in the HLA region of chromosome 6. This region contains several hundred genes known to be involved in immune response, and the role for HLA in pathogenesis of T1DM was reported by several studies (Grant etal., 2010; Mansoori Derakhshan, etal., 2015, Inaba. et al., 2022).

Material and Method:

Patients Group

Fifty Iraqi patients (females and males) with Type 1 DM enrolled in this study. They were attending the specialize center of diabetes (Central Teaching Hospital of peadiatric and Ebn –AL-Balade hospital-Baghdad) during the period between first of November / 2014 to 2022 . All patients were selected on the basis of criteria for diabetes which were used according to the American Diabetes Association 2007 guideline (ADA, 2007).

Control group

Thirty apparently healthy unrelated volunteers gender whose their gender and age3-13 matched the patients group, consisted of unrelated non-diabetic individuals according to the laboratory finding All of them had negative family history of DM

Samples Collection:-

Peripheral venous blood (5 ml) was collected via median cubical vein under good aseptic precautions using disposable, latex gloves and syringes from all subjects. . Whole blood samples was transferred to an EDTA tube and stored at-20ºC.

Isolation of Genomic DNA.

DNA genomic extracted from whole frozen blood by using ReliaPrep™ Blood g DNA Miniprep system kit (Pomega)USA.

Agarose Gel Electrophoresis

After genomic DNA was extracted, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (Sambrook and Russell, 2001).

Estimation of the DNA concentration and purity

The DNA Concentration of samples were estimated in ng/µl. by using Nanodrop result revealed 25.5-88.3ng/µl, and the purity detected by observing the ratio of optical density (O.D) 260/280 nm to detect the contamination of samples with protein. DNA purity for samples was between 1.7- 1.9 (Sambrook and Russell, 2001).

PCR Amplification

Components of PCR reaction

Detection of HLA Class II DQA1, DQB gene by PCR depending on the instruction of the company (IDT/USA), Table(1) shows the component of PCR reaction.

Table (1): PCR reaction mixture Component

Component	Volume
2Xkapa 2GRobust hot start polymerase	25 µl
Forward primer	2 µl
Reverse primer	2µl
DNA template	4 µl
D.W	14µl
MgCl2(1.5Mm)	3 µl
Total volume	50 µl

The components of the reaction were mixed by vortex and placed in a thermal cycler for amplification.

Primers:

Primers were designed by NCBI.

HLA-DQA gene

F-GTT CCT GAG GTC ACA GTG TT

R-CAG TGT TTC AGA AGA GGC TG

HLA-DQB gene

F- AGG ATT TCG TGT TCC AGT TTA AGG

R- CTC KCC KCT GCA AGR TCS

PCR program for amplification

Table 2: PCR program for DQA and DQB gene

HLA Class11 DQA				
No.	**Steps**	**Temperature(**°C)	**Time**	**Cycles**
1.	**Initial Denaturation**	94	3m	1
2.	**Denaturation**	94	16s	32
3.	**Annealing**	57	20s	
4.	**Extension**	72	20s	
5.	**Final extension**	72	10m	1
HLA class11 DQB				
No.	**Steps**	**Temperature** (°C)	**Time**	**Cycles**
1.	**Initial Denaturation**	94	3m	1
2.	**Denaturation**	94	21s	32
3.	**Annealing**	56	20s	
4.	**Extension**	72	20s	
5.	**Final extension**	72	10m	1

DNA Sequencing

The PCR products of fifteen of T1DM patients whom gave clear band after PCR analysis of the analyzed two genes with forward and Reverse primers were sent for sequencing in NICEM (Seoul International center For Environmental Management)

Results and Discussion

Demographic study:

Distribution of patients according to age:

Fifty Iraqi, T1DM patients were selected for this study and thirty control with age ranged from (3-13) years, (Table, 3)showed that the majority (56%) of them were at the age group of (7-10)years with mean age 8.75± 1.10 followed by(32%)11-13 age group with mean age9.37 ± 2.50 whereas the lowest (12%) at the age < 7years with mean age 4.96 ± 1.57 years and the mean age of control group 5.05 ±0.63, it seems that no significant differences between T1DM age groups. Another study carried out by Al-Agha etal.(2011) based on the analysis 547 of patients, the result that found out 162 (33.5%) were school children at age (7–12) years old, while the peak incidence T1DM 272 (56.2%) at age group 13-18 year old.

Table 3: Distribution of T1DM patients according to age

Age groups	Cases T1DM			Apparently Healthy controls			P value (t – test)
	n	%	Mean ±SD	n	%	Mean ±SD	
<7 years	6	12	4.96 ± 1.57	9	30	5.05 ± 0.63	0.88
7 – 10 years	28	56	8.75 ± 1.10	15	50	8.80 ± 1.14	0.89
11-13 years	16	32	12.12 ± 0.95	6	20	12.33 ± 0.81	0.64
Total	50	100	9.37 ± 2.50	30	100	8.38 ± 2.75	0.10

Distribution of patients according to gender:

All the patients and control distributed according to the gender and the result revealed that, 27 (54%) females and 23 (46%) males with ratio 1.2:1, (Table, 4). There are some studies showed that female's predominance among patients, as the study by AL Hassan etal.(2013) which showed about 42 (60%) of patients were females and 28(40%) males, female/male ratio was 1.5:1, also with other study by Al Ramahi (2009) has showed females/ male 1.1:1 with no significant differences in the gender distribution among T1DM patients. On contrast with other Iraqi studies were observed that females less than males as in Dawood (1998) 0.9:1; Salih (2008) 0.7:1 and abroad studies Shawkatova et al. (2006) 1:1.1 and Harjutsalo et al. (2008) 1:1.2.

Table 4: Distribution of T1DM patients according to Gender.

Gender	Cases T1DM n	%	Apparently Healthy controls n	%
Male	23	46	14	46.7
Female	27	54	16	53.3
Total	50	100	30	100

Distribution of patients according to age of onset:

The age of onset is a medical term referring to the age at which an individual acquires, develops or experience a condition or symptoms of a disease or disorder. In current study it was observed that the age of onset T1DM in 12 patients at age less than 5 years,

while in high number of patients (20) at 5-10 years and only 4 patients at age more than 10 years with highly significant difference ($p<0.0001$), while some of patients family can't remember age of onset their children(Table,5).

Table 5: Distribution of T1DM patients according to age of onset.

Age groups	Cases T1DM			(ANOVA) F-value
	n	%	Mean ±SD	
<5 years	12	24	3.66 ± 0.83	70.90
5 – 10 years	20	40	6.25 ± 1.07	
11-13 years	4	8	9.01 ± 4.29	
Not remembered	14	32		
Total	50	100	6.55 ± 2.76	

The distribution of patients according to family history of disease

The distribution of patients according to family history of disease explained in (Table, 6) it was shown that 12(24%) of fifty patients have positive family history of T1DM. This result similar to the result of study done by Al Hassan etal. (2013) who observe 15 (21%) of sixty patients with family history and to the finding of Tumba etal. (2014) who mentioned that 17 (28.3%) of sixty patients had family history and less than the percentage mentioned by AlRamahi (2009) which reached to 48(43.6%) of sixty patients.

Table 6: Distribution of T1DM patients according to History family

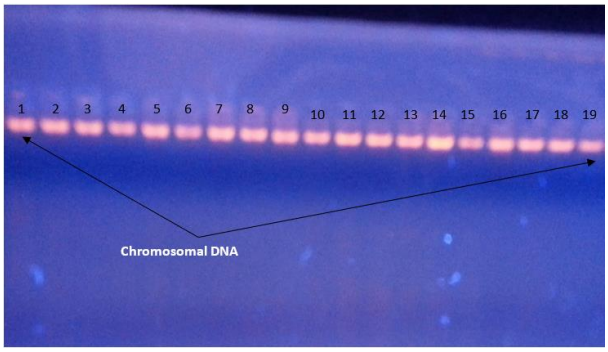
Family history	Cases T1DM	
	n	%
Positive	12	24
Negative	31	62
Not remembered	7	14
Total	50	100

Molecular study of T1DM:

DNA extraction:

The result demonstrated that the purity of the extracted DNA in all samples were sufficiently high for PCR analysis and appeared as compact separated bands by electrophoresis (Figure,1).

Figure 1: The genomic DNA purified from whole blood samples on 1% agarose gel at 70 volt/cm2 for 30min.



HLA Typing ClassII: DQA and DQB gene

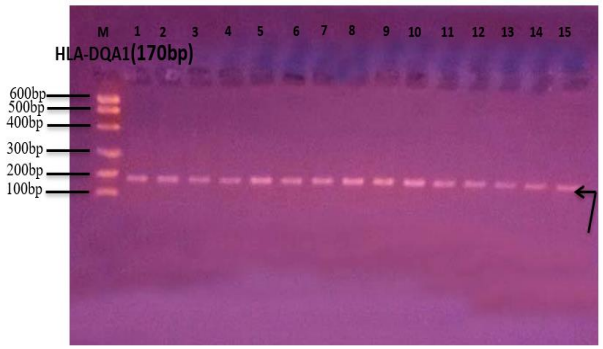
Whole genome linkage scan have shown that the (MHC)/HLA region on chromosome 6p21 contain the major genetic component of the T1DM (Cox et al., 2010).

The present work included polymerase chain reaction (PCR) amplifications of genomic DNA with primer pairs were designed for each gene of HLA typing ClassII:DQA1, DQB1 revealed products with length of fragment compared to ladder with size 170pb and 270bp respectively and sequencing Technique.

-HLA-DQA gene:

The result of amplification for DQA were showed with clear bands and the PCR product size fragment 170bp compared with ladder (Figure,2).

Figure 2: Detection of PCR product DNA bands of HLA-DQA gene with use of HLA-DQA primer (170bp). The amplified fragment were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 70 volt/cm2 for 90 minute. Photographed under UV light.



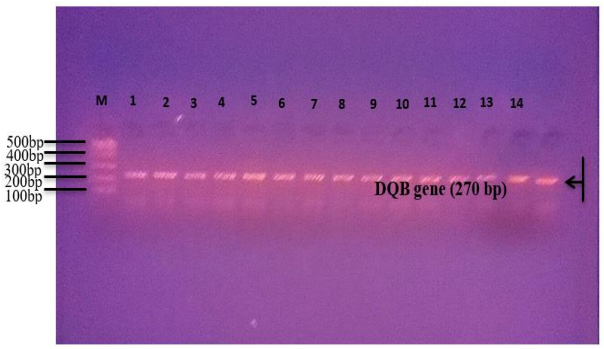
M: DNA ladder (1000pb)

Lane (1-15) amplified DNA of T1DM patients

HLA-DQB:

The result of HLA-DQB primer showed amplified fragment 270 bp as a clear band by electrophoresis on a 2% agarose gel at 70 volt/cm2 for 90 minute (figure, 3).

Figure 3: Detection of PCR product DNA bands of HLA-DQB gene with use of HLA-DQB primer (170bp). The amplified fragment were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 70 volt/cm2 90 minute. Photographed under UV light.



M: DNA ladder (1000pb)

Lane (1-15) amplified DNA of T1DM patients

Sequencing of HLA typing genes:

The sequencing was done for fifteen patients for each gene the result showed that variant that variants of DQA*0503 found in 4(26.67%)(as risk factor), DQA (wild type) found in 11(73.33%)(as a protective factor) O.R. 1.68 with highly significant difference (P<0.01) (Table 7).

Table (7): Distribution of T1DM patients samples study according to HLA-DQA (Class II).

HLA-DQA	Number	Percentage (%)	O.R.	Chi-square value	P-value
HLA-DQA	11	73.33	1.688	12.965 **	0.0001
DQA*0503	4	26.67			
Total	15	100%	---	---	---
**** (P<0.01). ** highly significant**					

The current study come in accordance with many studies were carried out in this field, like that conducted by Van der Auwera etal. (2002) who reported the DQA* 0501 associated with T1DM in Belgian population. Another study in Hungry by Eller etal. (2007) also noticed that DQA*0501 associated with T1DM.

The result of distribution of HLA-DQB sowed that DQB*02 in 7(46.67%) DQB*03 found in 1(6.67%)(as a risk factor), (Table 8) when DQB (wild type) appeared in 7 (46.67%)(as protective factor) O.R. 1.519 with highly significant difference(P<0.01)(Table, 8) .

Table (8): Distribution of T1DM patients samples study according to HLA-DQB (Class II).

HLA-DQB	Number	Percentage (%)	O.R.	Chi-square value	P-value
HLA-DQB*02	7	46.67	1.519	10.782 **	0.00214
HLA-DQB*03	1	6.67			
HLA-DQB	7	46.67			
Total	15	100%	---	---	---

**** (P<0.01).**
**** highly significant**

The association of specific HLADQB1 alleles and genotypes with T1DM susceptibility protection depends on the ethnicity and racial background of each population.

The present result similar to the result of many studies were done in different countries, it comes in accordance to other study in Iraq by Al-Ramahi (2009) which was confirmed high frequency of DQB*02 and DQB*03 in Iraq T1DM patients, other study in Arabian countries reached to same thing, Mossad etal. (2012) in Egypt was found the positive association with HLA-DQB*02 and HLA-DQB*03 in T1DM patients. Also Manan etal.(2010) from Saudi Arabia they were found DQB*02 and DQB*03 associated with T1DM.

Study by Shawkatova etal. (2006) from Slovakia noticed that DQB1*02 was associated with T1DM, also Eller etal. (2007) from hungry found DQB* 02 associated with T1DM. While, in Japan Katahira etal. (2008) mentioned that DQB1*04 and DQB1*03 were associated with T1DM in Japanese patients.

T1DM is considered a multifactorial condition with complex interactions between genetic and environmental factors (Shapira etal., 2010).

Conclusion:

The present study demonstrate that no significant difference between age and gender groups of T1DM patients, the highly incidence of T1DM occurrence was at the age group 7-10 years, it seems that association between the HLA Class II revealed by sequencing was finding the variation in DQA*0503,DQB*02,DQB*03 alleles. The inheritance of HLA genes associated with T1DM would involve the presentation of diabetic auto antigen to autoreactive T-cells, there by launching a T-cell activation cascade and the subsequent destruction of pancreatic β islet cells, the different of the result between studies may be related to background of population or more likely multiple etiologic bases for the disease, or may be related to the sample size.

Recommendation:

Based on the findings of the present study the following recommendation can be suggested:

1- Approaches need to be developed to store and use DNA-based HLA data more effectively, and then allow scientists to determine the role of HLA and additional T1DM candidate genes in the induction of autoimmunity and progression to diabetes.

2- Activate school health to start programs supported by ministry of health to screen primary and secondary schools for T1DM patients.

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