

The variant VNTR INS -23/Hph1 A>T (rs689) in the insulin gene has been related with T1DM in children.

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Abstract

T1DM is a complicated condition influenced with both inherited and biological conditions. Many studies have found a link between genetic variations and disease; however, the significance of specific gene loci is still unknown. The objectives of this paper were to see if there was a link between both the rs689 gene polymorphism and type1 diabetes in children. The A>T polymorphism in the INS-23/Hph1 gene is a significant risk susceptibility locus for T1DM in children, with T allele carriers having a 2.56 fold greater risk of getting the disease. Some of the conclusions are consistent with previous research, while others are not. The discrepancy in results could indicate that many mechanisms are at play, with central tolerance vs. peripheral tolerance being the most relevant aspect. This highlights the importance of doing susceptibility research on a local scale; results from one ethnic group or population cannot be applied to others. The study has other disadvantages, such as a small number of patients and a matched control group.

Keywords: T1DM, Insulin gene, PCR, RFLP, Gen polymorphism.

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Introduction

Diabetes is defined as a series of metabolic diseases that lead to the development of Diabetes Mellitus (DM) it is caused by a deficiency in insulin secretion, insulin efficiency, or both [1]. In type 1 diabetes, hyperglycemia arises when the pancreas fails to produce enough insulin or cells due to beta-cell insufficiency. That chemical effectively symbolizes non-diabetes, type 2 diabetes. Kind 1 diabetes is a type of diabetes that is Not Insulin-Dependent (NIDDM). One of the most deadly inherited autoimmune illnesses; it affects children in their youth and early adolescence. The loss of beta cells causes people with this kind to stop releasing the hormone insulin [2]. The basic element for T1DM pathogenesis is thought to be a mix of genetic susceptibility and environmental factors [3]. Alleles or genetic variants associated with T1DM provide vulnerability to or prevention from the syndrome depending on the environment [3]. HLA genes are the most important in determining T1DM vulnerability [4]. Outside of the Human Leukocyte Antigen (HLA) area, T1DM has been associated to more than 50 loci [5]. The genetic makeup of an individual, which includes a balance of susceptible and protective genes, determines the age at which T1DM develops. Genetic variants in the noncoding area of the Insulin (INS) gene (IDDM2) are second only to HLA in determining susceptibility to or prevention from T1DM [6]. At this position, a Variation Nucleotide Tandem Repeat (VNTR) mini-satellite can be found at the 5 terminus of the insulin gene [6]. VNTR size is divided into three classes: class I (26-63 repeats), class II (approximately 80 repeats), and class III (140-200 repeats). Insulin variants are linked to the VNTR region, so they can be

used as markers for these gene variants [7]. The 223HphI A>T SNP, a differential sequence in the INS gene, has just been found to be in high genetic linkage with the VNTR alleles, with the A allele related to the small (class I) VNTR allele and the T allele associated to the long (class III) VNTR allele [8]. The contribution of the HLA region and CTLA-4 has been the topic of study on the genetic basis of T1DM undertaken in AL-Muthanna, Iraq, as well as those conducted by the research team [8-10]. Just one previous study looked at the role of the INS gene in sensitivity to T1DM risk in a group of 25 participants. In the present study, the INS-23/Hph1 A>T (rs689) was investigated in diabetes children, non-diabetic siblings and parents, and also healthy controls. This, among other genetic data, could help predict whether a child born into a diabetic family will develop the disease, enabling for earlier treatment and improved care of diabetic youngsters. In Iraq, there are limited genetic investigations, particularly in the area of phenotype variation of genes that cause hereditary illnesses. For this study, the ARFLP technique was used to analyze the polymorphism of the genotype of individual using special primers and through which the allele is determined with PCR calculation) is the Cytokines whose repeats in the gene for samples studied [9].

Materials and Methods

Fifty blood samples were collected from children with diabetes-type 1 and 50 samples from healthy children (the standard group) from central children's hospitals and diabetes center in Al-Muthanna governorate Iraq for the period from February 2021 until April of the same year. The children's ages

ranged from 6-13 years old. Blood was collected in 5 ml blood collection tubes containing EDTA as an anticoagulant, which was used for DNA extraction. The samples were diagnosed by specialists in the diabetes unit at children's central hospital and the diabetes center. EasyPure® blood genomic DNA Kit used. The primers are prepared depending on the manufacturing

instruction by dissolving the lyophilized primers with TE (Tris-EDTA) buffer to make stock solution of concentration of 100 pmole/ml, On spinning down and stay overnight at 4°C , each primer's work solution was made by reducing the standard solution with TE buffer to reach final working solution (10 pmole/ml). The primers used in this study show in Table 1.

Primer	sequences	References
MPB	5'-FAGCAGG TCT GTT CCA AGG-3' R 5'AGCATCGAGTCGATCGCGGA '3	5

Table 1. Show primer and sequences used.

PCR master mix reaction was prepared by using (one taq quick-load) PCR Kit and this master mix done according to

company instructions as shown. The PCR setup steps show in Table 2.

Component	25 µl Reaction
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Template DNA	1.5 µl
OneTaq Quick-Load 2X Master Mix with Standard Buffer	12.5 µl
Nuclease-free water	10 µl
Total	25 µl

Table 2. The PCR setup steps.

Thermocycling conditions for a routine PCR show in Table 3.

Steps	Temperatures	Time
Initial Denaturation	94°C	30 seconds
30 Cycles	94°C	30 seconds
	55°C	60 seconds
	68°C	1 minute
Final Extension	68°C	5 minutes
Hold	4°C-10°C	∞

Table 3. Show thermo cycling conditions.

Genotyping of the INS-23/Hph1 A>T polymorphism PCR products from each sample was digested by 10000U of Hph I restriction. At 37°C for 18 hours, the amplicons were processed with Hph I restrict enzyme. Products were

electrophoresed on a 4 percent agarose gel then stained with ethidium bromide after digesting. The T and A alleles were separated by 231 plus 129 bp and 191 plus 129 bp bands, accordingly. Enzyme by using the protocol (Table 4).

Polymorphism	Restriction enzyme	PCR product (bp)	RFLP product (bp)	Mixture of restriction analyses				
				Enzyme	Buffer	ddH2O	PCR product	Total volume
INS -23/Hph1 A>T	10,000 units/ml (Hph I)	360	23,11,91,129	1 µl	5 µl	9 µl	10 µl	25 µl

Table 4. Show details of restriction enzyme.

Results

DNA extraction band from blood sample show in the flowing

picture of gel electrophoresis (Figure 1 and Figure 2).

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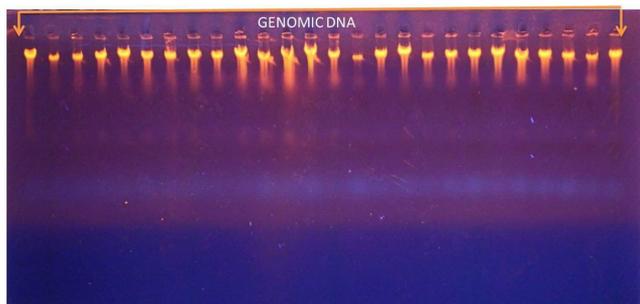


Figure 1. Ethidium bromide stained agarose gel electrophoresis (agarose 1.5% 30 min at 100 voltage). Appearance that displays genomic DNA that was extracted from blood samples.

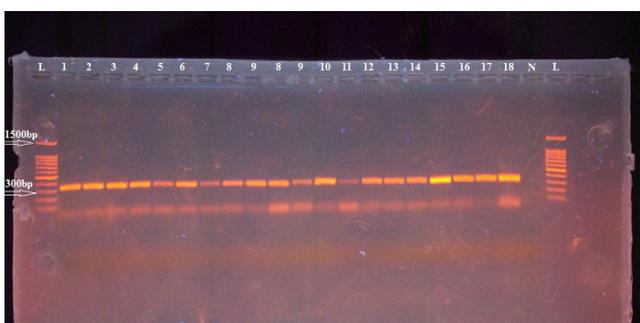


Figure 2. Gel electrophoresis for PCR product of (INS -23 primer) which show 360 bp primer TM at (55C), (Agarose 1.5% 15 min. At 100 voltage and then lowered to 70 volts, 60 min.). Visualized under UV light after staining with ethidium bromide. Lane L: DNA ladder (1500-100) bp, Lanes (1-18Z) represented positive results, lane N represent negative control.

In Table 1 shows the frequency of rs689 in the various groupings. The genotype frequency of the child's parents and non-diabetic siblings was comparable ($p=0.97$). In comparison to controls A was over-represented in the three family units, but TT and AT were over-represented in the controls ($p=0.0014$ and 0.0028 , respectively) (Figure 3).

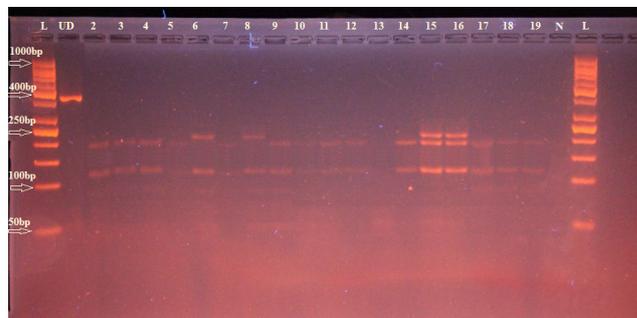


Figure 3. Agarose gels showing the enzyme-digested patterns of PCR products of INS-23/HphI A>T polymorphism. Electropherogram of ethidium bromide stained 4% agarose gel showing genotype pattern obtained with HphI restriction digest. Lane L: DNA ladder (50-1000) bp Lane UD refers to the undigested PCR-amplified (360 bp) fragment. Genotypes are characterized as AA (191 bp, 129 bp, and 40 bp) 40 bp fragments cannot be seen represented with (17-19) samples, AT (231 bp, 191 bp and 129 bp) represented with (15 and 16) samples, and TT (231 bp and 129 bp) represented with (6 and 8) samples. On the gel Lane (13) showed negative results, lane N represent negative control

Genotype and allele frequency shown in (Table 5).

Group	No	INS -23/ A>T genotype	Mixture of restriction analyses					
			enzyme	buffer	ddH2O	PCR product	Total volume	
INS-23/Hph1 A>T	10,000 units/ml (Hph I)	360	23,11,91,129	1 µl	5 µl	9 µl	10 µl	25 µl

Table 5. Genotype and allele frequency of INS -23 A>T in type 1 diabetes mellitus and control.

The AA allele was inherited from the mother in 34 patients and from the parent in 20 patients, the AT allele was received from

the parent in 23 instances and from the parent in 15 patients, and the TT allele was inherited from the parent in one incidence each (Table 6).

Groups	INS -23/ A>T genotype			Allele frequency	
	AA	AT	TT	A	T
Type 1 diabetes mellitus	27	21	2	0.75	0.25
Control	41	8	1	0.1	0.9

Table 6. Genotype and allele frequency of INS -23 A>T in type 1 diabetes mellitus and control according to Hardy Weinberg equilibrium for genotype frequency the results of our experiment as following.

In case to type 1 diabetes mellitus group allele frequency=0.999 which is not equal to a Hardy-Weinberg equilibrium $(A+T)^2=1$. SO this population is not is nearly

equal equal to a Hardy-Weinberg equilibrium based on the observations of above genotype frequencies.

Discussion

In the present study, the 223HphI A>T was evaluated in 91 diabetic children and their parents, as well as 96 healthy controls. Parents and siblings would be included since they share genotype(s) with the patients, at partly; the parents and siblings are not diabetic at this time. As according our data, T allele carriers are 2.56 times more likely to acquire T1DM [11]. The INSrs689 T variant was shown to be associated with a 2.44-fold higher risk, which is consistent with our findings. In other studies, homozygous TT genotype was linked to a 4.5-fold risk and 4.02, 4.4, and 6.96-fold hazards in three Israeli Jewish populations [12-14]. In addition to MHC (IDDM1), more than 50 Non-HLA genes have been linked to T1DM vulnerability [4]. The most efficient of them is the INS gene (IDDM2), which also plays a role in the evolution of other endocrine diseases [15].

Insulin variants have been used as a surrogate measure for gene changes in a number of studies [16-20]. Insulin variants are in linkage disequilibrium with the VNTR region. The homozygous genotypes AA and TA are linked to the risk and progression of type 1 diabetes. The type 1 genotype can be used as a risk indicator; results may correlate with immunologic findings of the TA genotype in serum of type 1 diabetes patients. IFN- γ levels are high and significant with a risk of developing IFN- γ compared to the standard sample, and in general genetic and immunological shows how important the IFN- γ gene [21-24]. It is less than homozygous for TT with type 1 disease, and the results also show the genotype Type I, ideas indicate the importance of the genetic model that was shown as a genotype associated with the risk of developing the disease. Type of genotype the results refer to the paragraphs of the studies and progress with them, as they agreed with the results of both [25-30]. In another study of the IFN- γ gene by blocking the IFN- γ receptor gene in Iranian patients with type 1 diabetes, the gene site has a negative association with the disease, and this site is considered as a protective indicator in the resistance of diabetes mellitus-type 1 in people with diabetes. The first type has the activity of IFN- γ .

The A T1DM-risk allele, which was related to lower insulin translation stages in the thymus and ineffective deletion of insulin-specific auto-reactive T cells in other studies, has been proposed as a mechanism for the association [31-33]. Several aspects of this explanation are debatable. To begin with, the genotype associated with lower insulin production in the thymus is simultaneously associated with increased insulin synthesis in the pancreas. This brings us to the second point to consider: In autoimmune diseases, central tolerance isn't the only mechanism at action; peripheral intolerance plays a role as well [33].

Conclusion

However, other studies have shown that overexpression of IFN- γ for the purpose of degrading Macrophage cells activates macrophage IFN- γ and acts as IL- β -cell toxicity. Besides this mechanical explanation, ethnic differences, such as the comparative genotype frequency in various groups, which

changes from one group to the next, have a significant impact on risk susceptibility associated with gene polymorphisms.

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