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ORIGINAL ARTICLE

Non HLA genetic markers association with type-1 diabetes mellitus

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Abstract The currently available data identified IDDM1 and IDDM2 as 2 susceptibility loci for type 1 diabetes (T1D). The major histocompatibility complex (MHC)/HLA region referred to as IDDM1 contains several 100 genes known to have a great influence on T1D risk. Within IDDM2, a minisatellite variable number of tandem repeats (VNTR) locus in the insulin gene (INS) promoter region is likely to represent the etiologic polymorphism. The aim of the present work was to study the association between genotypes and susceptibility to T1D among Egyptian diabetic children and their family members.

Twenty-five nuclear Egyptian families with 27 children having T1D, aged 3–14 years, their non-diabetic 44 sibs, aged 3–15 years and their parents were included in our study. All studied children were subjected to: detailed history and family pedigree. Thorough clinical examination and anthropometric measurements. Laboratory work up of diabetes including random blood sugar (RBS) and

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HbA_1C. Molecular genetics of INS was studied in four steps; nucleic acid purification, amplification, sequencing and haplotyping using flanking single nucleotide polymorphisms (SNPs) as surrogate markers for minisatellite alleles identification.

Analysis of variant repeat distribution among Egyptian families combined with flanking haplotypes revealed that all our diabetic children had class I alleles of INS; 9 had class IC+, 9 had class ID+ and 9 had class ID−, while all non-diabetic family members had class III alleles of INS. Therefore the three class I alleles were considered to be equally predisposing to T1D, while class III alleles are dominantly protective. There was significant positive correlations between body mass index (BMI) and both HbA_1C and AST liver enzyme among diabetic children with class IC+ but not other alleles; indicating that they need close monitoring of their diabetic control and liver functions beside following specific dietary regimens.

It can be concluded that all class I alleles (IC+, ID+ and ID−) are equally important susceptibility factors for T1D among Egyptian children, while class III alleles (IIIA and IIIB) are dominantly protective. It is concluded also that our diabetic children with class IC+ are an especially endangered subgroup of diabetics. Genotyping for INS-VNTR alleles is recommended for diabetic children as an important step of diagnostic and follow up regimens and for their non-diabetic family members for family counseling and early identification of potential diabetics. Further studies of INS-VNTR alleles and HLA haplotypes all over Egypt are recommended to define the Egyptian susceptibility loci for T1D and their relations to the clinical and laboratory findings as an important national programs.

1. Introduction

It is believed that most cases of type 1 diabetes (T1D) result from a T-cell dependent selective destruction of the insulin – producing pancreatic B-cells and subsequent irreversible insulin deficiency. T1D is caused by predisposing genetic factors in the presence of permissive environment [1]. At present, more than 20 regions of the genome are responsible for genetic susceptibility to T1D. The HLA region of chromosome 6 contains several 100 genes. Those most strongly associated with the disease are the HLA class II genes (i.e., HLA-DR, DQ, DP), also referred to as IDDM1. They contribute approximately 40–50% of the heritable risk for T1D [3]. Other genes in the central, class I [2] and extended class I regions may also increase T1D risk independent of HLA class II genes [4]. In addition to IDDM1, the insulin gene (INS), located on chromosome 11 p 15.5 designated as IDDM2 [5] and the CTLA-4 gene located on chromosome 2q31-35, are now known to influence T1D risk [6].

Within IDDM2, a minisatellite non transcribed variable number of tandem repeat (VNTR) locus in INS promoter region is likely to represent the etiologic polymorphism [7,8]. Differences in length occur in three discrete classes of VNTR alleles. THE shorter class of alleles (class I), predisposes to T1D, whereas the longer class of alleles named class III appears to be dominantly protective [9,10]. VNTR may bind transcriptional regulatory proteins related to the expression of insulin mRNA in the thymus depending on the sequence of the particular VNTR allele [11]. Class III variants appear to generate levels of insulin mRNA than class I variants. Such differences could contribute to a better immune tolerance for class III positive individuals by increasing the likelihood of negative selection for auto reactive T-cell clones [12]. Studies identified the variable number of tandem repeats as the T1D susceptibility locus (IDDM2), and classified alleles either by size or by flanking haplotypes [13–15]. The effect of INS appears to vary by ethnicity, with lesser effects in non-Caucasian populations [16].

The aim is to study different types and subtypes of the alleles of INS gene among Egyptian children with T1D and their diabetic and non-diabetic family members and also the relation to the clinical course of diabetes.

2. Patients and methods

2.1. Patients

Twenty-seven probands with T1D and their family members were studied. Probands (group 1) aged 3–14 years (11 males and 16 females) with mean age of 8.86 ± 3.93 years, were selected from our endocrine and genetic outpatient clinics and those admitted to pediatric department, Minoufiya university hospitals. Family pedigrees and detailed history suspected the diabetic family members of selected probands. Diabetes in probands and their affected family members was diagnosed according to WHO criteria after oral glucose tolerance test (OGTT) [17]. Forty four Sibs of type 1 diabetic patients derived from 25 families, were included (group II) (18 males and 26 females), their ages ranged from 3 to 15 years with mean age of 7.41 ± 4.27 years.

Healthy age and sex matched children were taken as a control group aged 3–14 years, with mean age of 3.91 ± 4.11 (group III), (10 males and 10 females) and 45 parents of diabetic children were included in our study (group IV), (21 fathers and 24 mothers), with mean age of 44.14 ± 7.82 years and 35.25 ± 6.11 years for fathers and mothers, respectively.

2.2. Methods

All studied diabetic children (after having written informed consents from parents) were subjected to the following.

A detailed history with stress on age of onset, type of presentation, and duration of diabetes beside consanguinity and family history and pedigree analysis.

A thorough clinical examination including 14 anthropometric measurements. Body mass index (BMI), pubertal assessment and full systemic examination.

Laboratory work up of diabetics including urine analysis, random blood sugar (RBS), complete blood count (CBC), liver and kidney function tests and glycosylated hemoglobin (HbA1C) were done. A ''trios'' design, selecting families for whom DNA was available from both parents and one or more offspring, was applied to clarify the imprinting effects [18].

Molecular genetics of insulin gene studied in our molecular genetics unit, pediatric department, Menoufiya university hospitals for a total of 136 members, of these 25 nuclear families (27 probands, 44 siblings, 45 parents and 20 control children).

First step was nucleic acid purification by genomic DNA extraction from peripheral blood using Bio-spin whole blood genomic DNA extraction kit ''Bioflux''. DNA was liberated, bounded to the Biospin membrane and then eluted after several washings.

Second step was amplification. In all PCR reactions 0.5 μg of genomic DNA was used. PCR primers used are: Sense primer: 5′-ACAGGCTGGACCTCCAG GTGCCTGTTCTG-3′. Antisense primer: 5′-TCGTCAGCACCTCTTCCTCAG-GACCAGC-3′. PCR was performed for 35 cycles (45 s at 95 °C, 45 s at 70 °C and final 5 min at 72 °C) in samples that suggested the presence of a class III VNTR allele; the Pst1 genotype was ascertained to confirm this, the PCR was performed for 35 cycles (45 s at 95 °C, 45 s at 68 °C, 1 min at 72 °C and final 5 min at 72 °C) and the primers used are: Sense primer: 5′-CTCTACCAGCTGGAGAACTA-3′. Antisense primer: 5′-GGCTGGTTCAAGGGCTTTAT-3′. The PCR and PCR digest products (for 12 h at 37 °C with 10–30 units of Pst1) were electrophoresed in 2% agarose gels after ethidium bromide staining.

The *third step* was sequencing; based on the almost complete linkage disequilibrium between INS–VNTR allele classes and the – 23 Hph1 single nucleotide polymorphism (SNP) in Caucasian, so the INS–VNTR genotype status by genotyping the – 23 Hph1 SNP as a surrogate for the VNTR [19]. The A to T polymorphism that disrupts the Hph1 site CCACT at nucleotide 2401 of INS was sequenced in all 136 family members of parent-child trios. The PCR primers were used to amplify a 574 – bp fragment from genomic DNA. Sequencing was done using the Big Dye Terminator on an automated DNA capillary sequencer (Model 3700; Applied Biosystems).

Detection of classes: much of the variations in VNTR length (repeat unit number of the oligonucleotide sequence: ACA-GGGGT(G-C)(T-C)GGGG) [20,21] and composition (repeat element sequence) were captured by dichotomizing VNTR alleles into two classes: the short class I alleles (26–63 repeats, average 570 bp) and longer class III alleles (141–209 repeats, average 2200 bp). The last step was haplotyping: the identity of the – 23Hph1 flanking site was determined by allele-specific PCR across the minisatellite using universal primer INS-1296 and allele specific primers INS-23+ and INS-23−. Polymorphisms at sites −2733 A/C, −2221 Msp1, +805Dra III, +1127 Pst1 and HUMTH01 was analyzed. Primers 3580-A and 3580-B were used to amplify a 188 bp region to type the +3580 $Msp1^{+,-}$ polymorphic site. Samples were electrophoresed for 2 h at 5 V/cm through 4% Metaphor (FMC Bio products, Rockland, ME) agarose gels in 0.5 × TBE buffer. Low haplotype diversity in the insulin gene region and strong linkage disequilibrium (LD) between VNTR allele structure and haplotypes spanning the insulin gene justified us to use flanking single nucleotide polymorphisms (SNPs) as surrogate markers for minisatellite lineages [21,22]. The – 23Hph1 SNP (rs689) denoted the class I/III subdivision; absence of the +3580Msp1 restriction site (rs2000993) distinguished the ID− haplotype from other class I haplotypes (IC+/ID+) [21–23]. Combining allele structure with the presence (+) or absence (−) of +3580$MSP1^{+/-}$ defined three class I subgroups: IC+, ID+ and ID−.

2.2.2.1. Statistical analysis

SPSS version 17 format was used to perform the descriptive and analytical statistics. As diabetic patients were 27 (less than 30) all the statistical procedures used the non parametric statistics. For descriptive purposes the median and the min–max values were used. For comparison of the three subgroups the Kurskal Wallis test was performed, cross tabulation using Chi square was also used to test the association between cat goral variables. Finally the non-parametric correlation (Spearman correlation) was used to test the correlation between the quantitative variables. Alpha error was set to 5%.

3. Results

The results of this study illustrated in Tables 1–6 and Figs. 1–3.

Classes and subclasses of INS allele in patients, sibs and controls (Table 1). Minisatellite VNTR–CR analysis of INS gene demonstrated that all our diabetic children had class I alleles and all their family members as well as controls had class III alleles, except three mothers had class I allele.

Demographic data family history of diabetes as well as positive consanguinity were noticeably high among diabetic children 92.3%) and (62.9%), respectively. Age of onset of T1D ranged between 1 and 12 years (median age was 7 years). Two cases started in the first year of life and 4 cases started up to 2 years of age. The present work showed also a high percentage of the occurrence of diabetic ketoacidosis as the initial presentation of T1D (54.8%). The duration of T1D among our diabetics was 1–5 years (median = 2 years). There was delayed onset of puberty in 6 patients (22.22%). The clinical assessment of our patients showed normal body system examination. Anthropometric measurements and body mass index were within normal ranges appropriate for age. There is no significant difference between age at onset of diabetes, sex of the patients, family history, positive consanguinity, duration of type 1 diabetes, birth weight, pubertal development, anthropometric body measurements and the subclasses of INS gene (Table 2).

Laboratory data (Table 3), hepatic and renal function tests and counts of WBCs and platelets were quite normal for age and sex without significant differences between diabetics and control children and also among diabetic children of different subclasses of INS. Mean levels of Hb were slightly decreased among all diabetics (median = 10.85 g/dl) ($p > 0.05$).

On the other hand both RBS and HbA_1C (medians 212.5 mg/dl and 9.95%, respectively) were significantly increased among our diabetic children reflecting their poor diabetic control and necessitating better control regimens. Glucosuria was present in all our diabetics except for 6 patients, while urinary tract infection was present in 5 out of the 27 diabetic children (18.51%).

Table 1 Comparison of classes and subclasses of INS gene alleles in patients, sibs and controls.

Genotyping	Diabetic group (n = 27)		Sibs group (n = 44)		Control group (n = 20)		X^2	p Value
	%	No	%	No	%	No		
Class I:								
IC	33.3	9	0	0	0	0	26.37	<0.001*
ID+	33.3	9	0	0	0	0	26.37	<0.001*
ID−	33.3	9	0	0	0	0	26.37	<0.001*
Class III	0	0	100	44	100	20	66.16	<0.001*
IIIA					10	2		
IIIB					15	3		

Five controls were genotyped for subclasses III/A and B.
* $p < 0.05$ = significant.

Table 2 Demographic data of all studied diabetic children in relation to their subclasses.

	IC (n = 9)			ID+ (n = 9)			ID− (n = 9)			X^2	p-Value
	$X^- \pm SD$	%	No	$X^- \pm SD$	%	No	$X^- \pm SD$	%	No		
Age in year	8.5 ± 3.62			8.0 ± 4.0			10.13 ± 36			1.19	>0.05
Sex: males		33.3	3		37.5	4		37.5	4	0.04	>0.05
Birth order											
1st		44.4	4		44.4	4		55.5	5	5.6	>0.05
2nd		2.22	2		11.1	1		22.2	2		
3rd		11.1	1		11.1	1		11.1	1		
4th		22.2	2		33.3	3		11.1	1		
Consanguinity +ve		55.6	5		55.6	5		73.6	6	0.26	>0.05
Perinatal history											
• Normal		66.7	6		66.7	6		55.6	5	2.11	>0.05
• Low birth weight		22.2	2		12.5	1		33.3	3		
• IDM		11.1	1		22.2	2		11.1	1		
Family history +ve		66.7	6		88.8	8		33.3	3	9.81	>0.05
Other sibling affection +ve		0	0		22.2	2		22.2	2	1.22	>0.05
Type of presentation DKA		66.7	6		66.7	6		55.6	5	0.29	>0.05
Age at onset	6.72 ± 2.28			6.0 ± 3.57			7.75 ± 3.79			1.05	>0.05
Duration of illness	2.5 ± 0.87			2.0 ± 1.0			2.38 ± 0.58			2.66	>0.05

DKA = diabetic ketoacidosis.
IDM = infant diabetic mother.

Correlation studies of demographic, anthropometric and laboratory findings of all studied diabetic children; the only significant correlation was the logic positive correlation between the 2 indices of diabetic control i.e., random blood sugar and HbA_1C (p = 0.007). Correlations among subclass IC+ diabetic children were significant, there is positive correlation between BMI and liver functions (p = 0.05) and HbA_1C (p = 0.042); indicating the adverse effect of obesity on liver function and diabetic control.

Family history, none of the fathers of diabetic children suffered from diabetes mellitus while 3 mothers (12.5%), suffered from DM, one of them had T1DM and two mothers had T2DM. Eleven children (44%) of the diabetic group had positive family history of T2D, from paternal side and 3 children (12%) of diabetic group had positive family history of T1D and 5 children (20%) had positive family history of T2D from maternal side (Table 4).

Table 5 demonstrates classes and subclasses of INS gene among parents of diabetic children.

Twelve mothers having the genotype of the heterozygous class I/III allele had diabetic children with genotype class I subgroups as: 6 mothers (50%) had diabetic children with genotype of IC subclass, 1 mother (8%) had diabetic child with subclass ID+ and 5 mothers (42%) carried the heterozygous class I/III had diabetic child with subclass ID−. While 8 fathers, who were heterozygous for INS VNTR alleles, had diabetic children with class I subgroups as: 2 fathers (25%), had diabetic children with subclass IC, 3 fathers (37.5%) had diabetic children with ID+ subclass, and 3 fathers (37.5%) had diabetic children with ID− subclass. Higher percentages of diabetic children with ID− subclass were detected in mothers than in fathers who were heterozygous for INS VNTR allele as shown by percentage value of (42%) in mothers in comparison to (37.5%) in fathers (Table 6).

Table 3 Comparison between genotyping (class I subclasses), some clinical and laboratory data among diabetic group.

		IC (n = 9)	ID+ (n = 9)	ID− (n = 9)	*F* test	*p* Value
Random blood sugar (mg/dl)	X ± SD	258.33 ± 87.34	215.63 ± 69.31	230.75 ± 59.95	0.74*	< 0.05*
Glycosylated hemoglobin (%)	X ± SD	10.63 ± 1.48	10.21 ± 2.19	11.24 ± 1.12	0.78*	> 0.05
Liver function tests						
• ALT (mg/dl)	X ± SD	16.33 ± 6.58	16.87 ± 4.22	16.37 ± 3.99	0.65	> 0.05
• AST (mg/dl)	X ± SD	21.89 ± 7.62	18.75 ± 4.43	17.63 ± 1.99	0.63	> 0.05
Kidney function tests						
• Blood urea nitrogen (mg/dl)	X ± SD	16.89 ± 6.55	18.13 ± 1.36	18.38 ± 5.29	0.33*	> 0.05
• Creatinine (mg/dl)	X ± SD	0.82 ± 0.07	0.79 ± 0.12	0.83 ± 0.12	0.78*	> 0.05
Complete blood picture						
• Hb (gm/dl)	X ± SD	11.1 ± 0.44	11.4 ± 0.82	11.08 ± 0.41	1.21*	> 0.05
• WBC'S ($\times 10^3/mm^3$)	X ± SD	6.63 ± 3.84	7.8 ± 3.52	7.8 ± 3.72	0.55	> 0.05
• Platelets ($\times 10^3/mm^3$)	X ± SD	226.22 ± 16.72	228.37 ± 14.02	230.75 ± 15.82	0.22*	> 0.05

* *p* Value < 0.05 = significant.

Table 4 Family history of DM among parents and siblings of diabetic children.

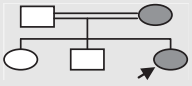
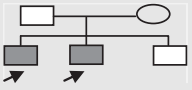
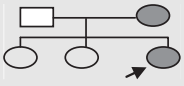
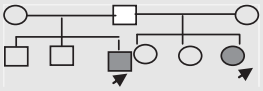
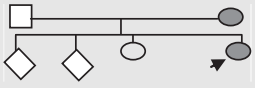
Family number	Family history of DM			Family pedigre
	Siblings	Parents		
		Fathers (mean age in years 44.14 ± 7.82)	Mothers (mean age in years 35.25 ± 6.11)	
10	−ve	−ve	T2D	
11	+ve	−ve	−ve	
22	−ve	−ve	T2D	
24	+ve	−ve	−ve	
25	−ve	−ve	T1D	

Table 5 Frequency distribution of INS VNTR alleles among parents of diabetic group.

INS genotyping	Fathers of diabetic patients (*n* = 21)		Mothers of diabetic patients (*n* = 24)		X^2	*p*-Value
	%	No	%	No		
INS VNTR						
Class I	0	0	12.5	3	2.81	> 0.05
Class III						
IIIA	61.9	13	7.5	9	2.67	> 0.05
IIIB		2		1		
		1		1		
Class I/III						
Heterozygosis	38.1	8	50	12	0.64	> 0.05

N.B: Subclassification of class III into A and B were done only for 5 of the parents.

Table 6 Relation between class I/III alleles of parents and subclasses of diabetic patients.

Diabetic group	Mothers I/III (n = 12)		Fathers I/III (n = 8)		X^2	p-Value
	%	No	%	No		
IC (9)	50	6	25	2	1.25	>0.05
ID+ (9)	8	1	37.5	3	2.55	>0.05
ID− (9)	42	5	37.5	3	0.03	>0.05

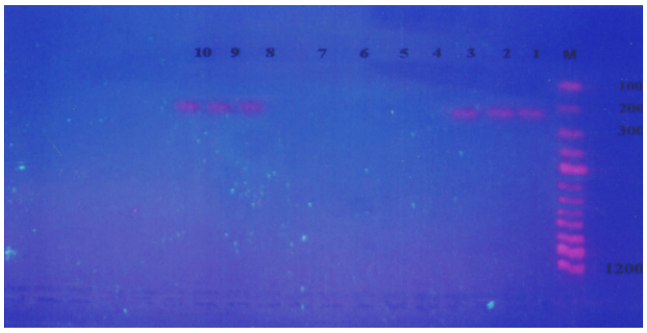


Figure 1 Gel electrophoresis of the PCR of the INS gene for diabetic, sibs and control groups. Lane M: DNA ladder of 100-bp, starting from 100 to 1200 bp (on the right side). Lanes 1, 2, 3, 8, 9, 10: indicated samples from diabetic children, positive for class I genotyping as shown by the presence of corresponding bands on gel along the rum (at ~200 bp). Lanes 4–6: indicated samples from sibs of diabetic children. No bands were visualized on gel. Lane 7: indicated sample of PCR from a normal child.

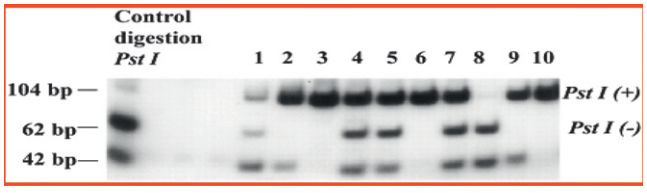


Figure 2 Genotypes of different Egyptian diabetic children at the *INS* 3′ *Pst*I RFLP. In most, the presence of the *Pst*I restriction site indicates the presence of a class III allele visualized at the VNTR on the chromosome, whereas the absence of the restriction site indicates the presence of a class I allele on the chromosome. The *numbering* at the *top* of each lane indicates different samples.

VNTR consensus	: ACA	GGGG	TGT	GGGG
A	: ---	----	-C-	----
B	: ---	----	-CC-	----
C	: ---	----	-CCC	----
E	: -T-	----	----	----
F	: -T-	----	----	GTGG
H	: ---	----	-CCC-	-A--

Variant repeat distribution at the insulin gene for all patients with class I

Figure 3 Sequence of various repeat elements present in VNTR regions.

4. Discussion

To our knowledge, this is the first study to be done in Egypt on diabetic children.

Whole genome linkage scans have shown that the major histocompatibility complex (MHC)/HLA region on chromosome 6p21 contains the major genetic component of the disease (IDDM1) [24,25]. Another established disease locus, IDDM2, has been mapped to chromosome 11p15.5 and carries a relatively more modest but clearly defined genetic effect [5,26]. The insulin gene variable number tandem repeats locus (INS-VNTR) is the best candidate for T1D susceptibility locus; IDDM2 [27]. The INS-VNTR locus affects expression of the insulin gene and its precursors in the thymus, consistent with a model in which either positive or negative selection of auto reactive T-cell clones against epitopes from preproinsulin (PPI) occurs [28–31]. Overall the biological pathways leading to T1D are not fully understood and the nature of the interaction of the products of HLA class II and INS-VNTR in the disease process is still unclear. Indeed, the functional consequences of INS-VNTR variation in T1D susceptibility might include extra-thymic immunological and metabolic effects [1,32]. Furthermore, the joint action of INS gene and HLA variation might also be influenced by additional genetic and environmental factors, which could vary in different populations [1]. The apparent role of INS-VNTR in susceptibility to T1D may be modified by neighbouring haplotypes and therefore IDDM2 could be a multi-locus etiology [21]. The present work analyzed INS-VNTR distribution within a number of Egyptian families and combined this with flanking haplotypes to define different ancestral allele lineages. Previous studies identified INS-VNTR as T1D susceptibility locus and classified its alleles either by size or by flanking haplotypes into a shorter class I variant predisposing to T1D and a longer class III variant which appears to be dominantly protective [9,10]. The effects of INS-VNTR alleles vary by ethnicity [16]. The present study revealed by analysis of variant repeat distribution among different Egyptian families combined with flanking haplotypes that all our diabetic children had class I alleles. Nine of them had subclass IC; 9 had subclass ID+ and 9 had subclass ID−. While all their non-diabetic family members had class III (IIIA and IIIB). Linkage disequilibrium between the INS minisatellite and HUMTH01 was analyzed. HUMTH01 has 5 common alleles composed of 6 (allele Z – 16) to 10 (allele Z) tandem repeats of a TCAT tetramer. Haplotypes of the 5 subclasses of the minisatellite were each dominated by a different allele of HUMTH01 confirming that each subclass represented a distinct ancestral lineage that has retained linkage disequilibrium over a region of at least 9 kb. The greatest breakdown of LD was within the ID− lineage, where ID− haplotypes were associated with both allele Z– 16 (59% of

haplotypes) and allele Z (41% of haplotypes) at HUMTH01 [33]. In general, class I alleles were found to be equally predisposing to T1D with the exception of ID− alleles which are protective at certain circumstances. ID− alleles are typically larger than either IC or ID+ alleles. A size threshold was therefore alleged, above which class I alleles are protective. Alternatively, protection was supposed to be due to variant repeat distribution within the insulin minisatellite (ABA motif 10 repeats, 5′ CAC block, a central FAC block and a 3′ F repeat). However, ABA repeats are shared in all IC, while other repeats are shared by most ID+ alleles, suggesting that ID− protection is not mediated by variant repeat composition or distribution. A strong etiological candidate for ID− protection is therefore the flanking haplotype. All ID− alleles lack the $+3580\text{Msp1}^{+/-}$ polymorphic site within the first intron of IGF2 >4 kb downstream of the minisatellite, which is present in 99.6% of all other haplotypes. It is therefore suggested that IDDM2 may have a multi-locus etiological basis, with the putative effects of the secondary polymorphism depending on both parental genotype and gender [7,22]. The 3 subclasses (IC, ID+ and ID−) were equally presented among children with type 1 diabetes. Failure to discriminate predisposing and protective effects of the three subclasses could be due to the small sample size or to racial genotypic differences. The complete association in 100% of our children with T1D with class I alleles in contrast to occurrence of class III alleles in T1D in 2.5% of diabetic children in a German study [34] and 24% of a Czech population [35]. It is to be stated that our research attributed a definite predictive role in T1D for genotyping of the insulin gene region polymorphism with class I alleles in all our children with T1D in contrast to Undlien et al. [16] who found that it is not a definite tool in the prediction of T1D. On the other hand, Cinek et al. [35] stated that the insulin gene genotyping confers important information on T1D risk in Czech population and they recommended using it in determining the disease risk along with the HLA – DQ typing. The absence of classes IIIA (protective haplotype) and IIIB (very protective haplotype) among diabetics and being the only haplotypes detected among our non-diabetic family members confirms the protective role of these haplotypes among Egyptian families. Differences in repeat-type distribution and composition may account for this [7]. H-type repeat may protect against disease by increasing insulin gene transcription through the binding of the pur – 1 transcription factor [36], although its presence in IIIA (protective haplotype) but not IIIB (very protective haplotype) alleles excludes it as the sole determinant of protection. A-type repeats elevate insulin gene transcription. However; the higher frequency and copy number of A-type repeats found in IIIA alleles compared to the IIIB alleles is against this functional role. However, in IIIB alleles 5/6 repeats closet to the insulin gene were A-type compared to 0/6 in IIIA alleles, so repeat distribution may be a more important factor than absolute composition [7]. F-type repeats within each lineage were conferred a protective role [22]; however, the present study found no correlation between the number of F-type repeats and the level of protection; for example there was no detectable difference between the effects associated with IC (2F-repeats) and ID+ (4F repeats) alleles. As regards demographic, clinical and laboratory findings among our children with T1D and their relations to INS subclasses. The present work found a markedly high familial occurrence of T1D (64%) irrespective of INS subclasses which may be attributed to our rural cultural habit of marriage of relative (documented by high +ve consanguinity in 56%), It was also observed that two families had other siblings affected with T1D (*Families No. 11*, *24*) beside the genetic basis of T1D as a disease and also the racial differences. A study from Saudi Arabia [37], found +ve first degree family history for T1D in 28% of patients. A possible explanation can be deduced from Snehaltha et al. [38] who stated that there is evidence that genetic and ethnic variations are present in the familial occurrence of T1D. There was also a high occurrence rate of diabetic ketoacidosis as the initial presentation of T1D among our diabetics (68%) regardless of their subclass of INS nearly similar (25%) to Munk [39]. The present work showed no association between age at onset of T1D as insulin gene variation in agreement with Desai et al [21]. On the other hand there was contradiction between the present study and previous studies as regards the insignificant differences of the birth weight [40–44] of our diabetics and also their delayed pubertal onset [45]. These contradictions may be explained by the smaller sample size of our study and/or racial differences. The present work showed that BMI is more sensitive than all anthropometric measurements to be affected earlier in T1D and that hemoglobin level is the most vulnerable hematological parameter to be altered in T1D. Likewise, urinary tract infection was found in a considerable number of our children with T1D. Correlation studies done among our diabetic children put subclass IC as the subclass with a considerable risk of obesity (high BMI), poor diabetic control and impairment of liver function.

The current study showed that fathers, free from either T1D and T2D, showed that 13 of them (61.9%) were positive for INS class III allele. Eight fathers (38.1%), were positive for the heterozygous class I/III. As regards mothers of diabetic patients, 3 diabetic mothers (12.5%), were positive for the predisposing class I allele. Nine mothers (37.5%) were genotyped under class III allele, and 12 mothers (50%) were positive for the heterozygous class I/III.

As regards parent-offspring relationship, 5 diabetic patients (42%) genotyped for ID− subclass, were born to heterozygous I/III mothers and 3 diabetic patients (37.5%), who were genotyped for ID− sub class, were born to heterozygous I/III fathers. Although this may be contrasted with the previously mentioned that Class ID− allele carries protection when transmitted from heterozygous fathers. *Dos Santos et al stated a* restriction of ID− protection to transmission from fathers [46,47].

This does raise the possibility that ID− alleles may be more protective against T1DM than class III alleles transmitted from *ID−/III* heterozygous fathers.

To clarify that protective effect of the ID− lineage was a property specifically of allele 814, Le Stunff et al. [47] stated that molecular genetic studies divided all ID− alleles into those of 42 repeats and those of other sizes and analyzed the risk of transmission into offsprings.

Dos Santos et al. [46] reported that levels of protection associated with ID− alleles were indistinguishable between ID−/III A and ID−/IIIB fathers, indicating that the ID− effect is not significantly modulated by differences between the lineages of untransmitted class III alleles.

Meigs et al. [48] identified that this potential evidence of imprinting effect about the paternally transmitted alleles altered with presence of maternal hyperglycemia, that is, especially if the mother had type 1 DM. However, this parent of

origin effect has not been replicated in an independent family data set and until this is achieved its existence and properties remains speculative.

In fact our results supported that the molecular structure of the INS VNTR allele, not only its overall length is associated with variations of insulin secretion. It may be important to test the ramifications of this observation for class I association with type 1diabetes susceptibility [49].

In analogy, to the genes for sickle cell anemia, Tay-sachs disease and thalassemia, which are examples of population-specific genetic markers for susceptibility, INS class I, result in higher incidence of T1D, and marks T1D susceptibility, mediated by both composition and variant repeat distribution. It is important to note that it is the contrasting frequencies of susceptibility alleles at different susceptibility loci in the parental populations that determine the rising disease incidence in the offspring [50].

It is concluded that: (1) Class I alleles of INS are important susceptibility factors for T1D among Egyptian children (children with class I alleles are at a greater risk to develop the disease). (2) Our diabetic children having subclass IC are more vulnerable than those with ID+ and ID− alleles and need strict diabetic control and follow up. (3) Class III alleles are dominantly protective against T1D among Egyptians.

It is recommended to include genotyping for INS-VNTR alleles as an important step of the family counseling of T1D and as an essential part of diagnostic and prognostic work up of diabetic children. It is also recommended to periodically check and early manage obesity, anemia, poor diabetic control, liver function affection and urinary tract infection among all our diabetic children especially the more vulnerable diabetic children with class IC alleles. It is to be recommended to determine INS-VNTR alleles beside the HLA haplotypes among diabetic children and their relatives all over Egypt in relation to clinical findings as an important national project.

The authors declare that there is no conflict of interest.

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