The Egyptian Journal of Medical Human Genetics (2014) **15**, 265–272

Ain Shams University

The Egyptian Journal of Medical Human Genetics

www.ejmhg.eg.net
www.sciencedirect.com

ORIGINAL ARTICLE

The association of polymorphic sites in some genes with type 1 diabetes mellitus in a sample of Egyptian children

Ahmed A. El-Kafoury [a,*], **Medhat Haroun** [b], **Amira Mohamed Embaby** [b], **Ali Salem Dawoods** [b]

[a] *Department of Pediatrics, Faculty of Medicine, Alexandria University, Egypt*
[b] *Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Egypt*

Received 24 April 2014; accepted 15 May 2014
Available online 23 June 2014

KEYWORDS

Genes;
Genomic DNA;
Single nucleotide polymorphism;
Type 1 diabetes mellitus

Abstract *Background:* The major histocompatibility complex (MHC) genes have been implicated as the major genetic component in the predisposition to type 1 diabetes mellitus (T1DM). Other loci outside the MHC had also been reported to contribute in the susceptibility of T1DM. The aim of this study was to examine the role of some variants of polymorphic sites in some genes associated with T1DM in a sample of Egyptian children.

Patients and methods: 60 patients with T1DM from the diabetes clinic at Alexandria University Children's Hospital, and 60 healthy individuals were enrolled in this study. Genomic DNA was extracted using isopropanol precipitation method. *Interleukin 18* (*IL-18*), *interleukin 10* (*IL-10*), *vitamin D receptor* (*VDR*), *protein tyrosine phosphatase non-receptor type 22* (*PTPN22*) and *cytotoxic T-lymphocyte antigen-4* (*CTLA-4*) were genotyped.

Results: The findings obtained from logistic regression analysis suggest that the *IL-18* single nucleotide polymorphisms *SNP-137 G > C* (*rs#187238*), the *VDR Fok1 SNP T > A* (*rs#2228570*) and the *SNP-1123 C > G* (*rs#2488457*) in *PTPN22* gene showed a significant difference between patients and controls ($P = 0.026$, 0.030, and 0.003, respectively). The genotype distributions of *PTPN22 SNP-1858*, *CTLA-4 SNP 49*, *IL-10 SNP-819*, *IL-18 SNP-607*, and *VDR BsmI SNP G > A* did not show any significant difference.

Conclusion: The *IL-18 SNP-137 G > C* (*rs#187238*), *VDR SNP-Fok1 T > A* (*rs#2228570*), and the *SNP-1123 C > G* (*rs#2488457*) in *PTPN22* gene may have an effect on the occurrence of

* Corresponding author. Address: Alexandria University Children's Hospital, 77 Al Fath Street, Fleming, Alexandria, Egypt. Tel.: +20 1222989642, +20 35830023; fax: +20 34876099.
E-mail address: aaakafouri@hotmail.com (A.A. El-Kafoury).

Peer review under responsibility of Ain Shams University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.ejmhg.2014.05.002

T1DM in Egyptian children. Further large-scale, population-based, case-control studies are needed.

1. Introduction

Type 1 diabetes mellitus (T1DM) is a common, chronic metabolic disorder characterized by hyperglycemia as a cardinal biochemical feature and disturbances of carbohydrate, fat and protein metabolism, associated with absolute or relative deficiencies in insulin secretion and/or action [1]. This is usually due to autoimmune destruction of the pancreatic beta cells (type 1A) [2].

The incidence varies from more than 40 per 100,000 children in Finland to less than two per 100,000 in Japan. A rise in the numbers of children and adolescents with T1DM has been observed since the mid-1950s, both in high- as well as low risk countries. The current global prevalence rate is 0.025% for children under 15 years of age, with the average increase in the annual incidence rate for this age group being 3% [3,4].

T1DM is a complex polygenic disorder and cannot be classified strictly by dominant, recessive, or intermediate inheritance, making identification of disease susceptibility or resistance genes difficult [5,6].

The major histocompatibility complex (MHC) genes have been implicated as the major genetic component in the predisposition to T1DM but other genes are likely to be involved such as *Interleukin-10* (*IL-10*), *Interleukin-18* (*IL-18*), *cytotoxic T-lymphocyte antigen-4 gene* (*CTLA-4*), Toll-like receptors 2 (*TLR2*), *insulin gene* (*INS*), protein tyrosine phosphatase non-receptor 22 (*PTPN22*), *interleukin 2 receptor alpha* (*IL2RA*/*CD25*), *glutamate decarboxylase 2* (*GAD2*), *vitamin D-receptor* (*VDR*) *gene* and others. These loci have all been proved important in the pathogenesis of autoimmunity when globally considered, whereas the insulin gene is a disease-specific T1DM predisposition locus [5,6].

At least 20 different chromosomal regions have been linked to T1DM susceptibility in humans, using genome screening, candidate gene testing and studies of human homologues of mouse susceptibility genes [7].

Since 2001 a significant number of genome-wide association (GWA) studies have been reported. Data from The International Type 1 Diabetes Genetics Consortium (T1DGC), collected through multiple GWA studies and large scale meta-analyses, identified more than 40 loci that affect the previously reported as regions associated with T1DM susceptibility. Eighteen additional regions showed significant association with T1DM and several of them contain new candidate genes of possible relevance to T1DM (*IL19*, *IL20*, *GLIS3*, *CD69* and *IL27*). Most of the listed genes mediate the immune response, some exert their functions in the process of destruction of pancreatic β cells and some have a dual role [8]. Additional functional studies provided evidence of causality of several genes within established loci, such as several cytokines and their receptors, immunomodulatory molecule. However, for the majority of associated regions the most likely causal gene still needs to be identified [9].

The aim of this study was to examine the role of some variants of polymorphic sites in some genes associated with T1DM in a sample of Egyptian children.

2. Patients and methods

A total of 60 patients with type 1 diabetes (25 male/35 female) from the Diabetes Clinic at Alexandria University

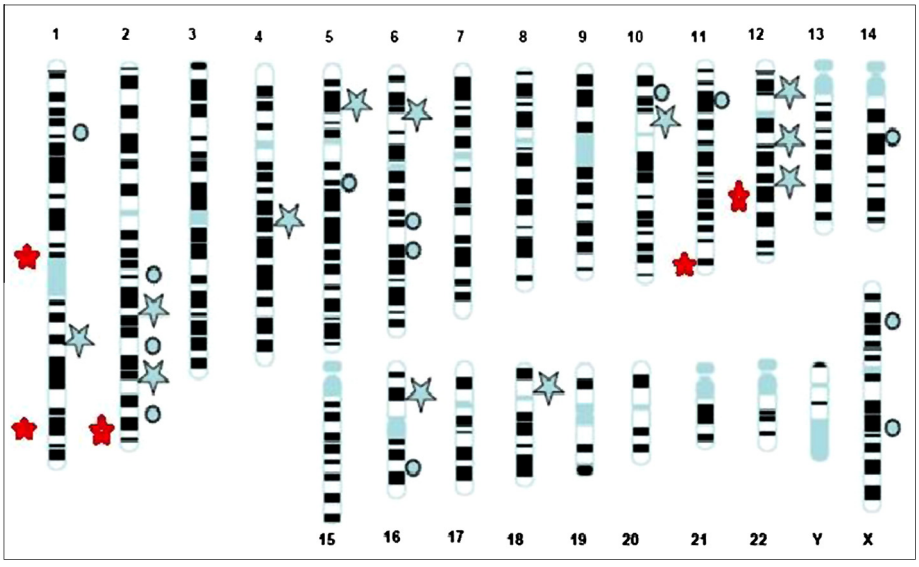

Figure 1 The type 1 diabetes risk gene loci paced over the genome. Stars are the loci that have shown evidence of association to T1DM, dots are the loci identified in linkage studies [10]. The red stars are genes loci that were investigated on chromosomes.

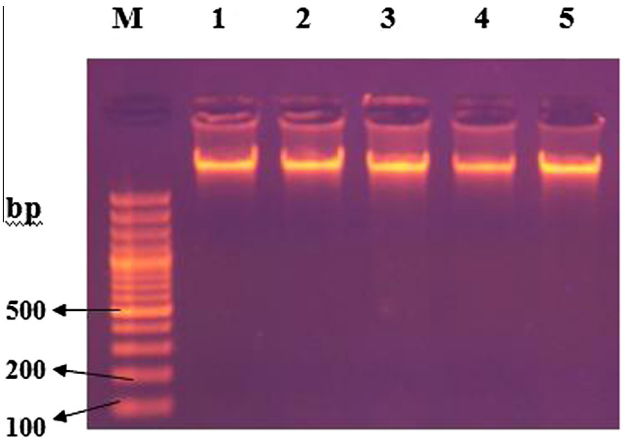

Figure 2 1% agarose gel electrophoresis of DNA extracted from whole blood samples of some subjects under study (lanes 1–5). Lane (M) represents 100 bp DNA ladder.

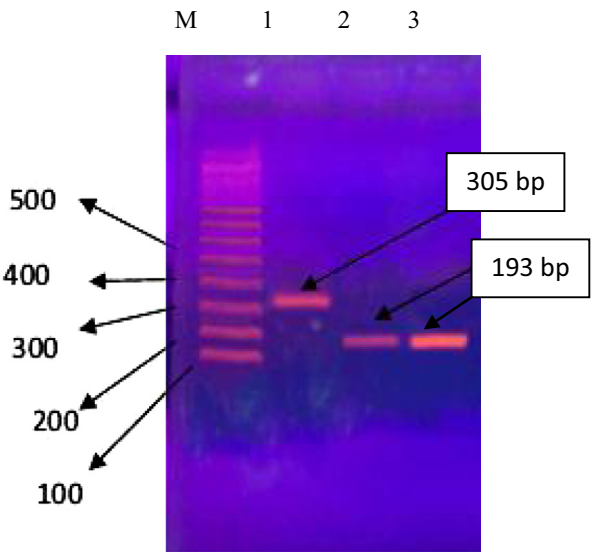

Figure 3 2% agarose gel electrophoresis for allele specific PCR for *Il-18* SNP-607 C > A (rs#1946518). M: 100 bp DNA ladder from GeneDire*X*®. Lane 1: PCR product upon using control forward primer, lanes 2 and 3: PCR products, represent heterozygous upon using allele specific C primer and allele specific A primer respectively, lane 2: PCR products represent homozygous upon using allele specific C primer and lane 3 will be empty, lane 3: PCR products represent homozygous upon using allele specific A primer and lane 2 will be empty.

Children's Hospital, (El-Shatby) and 60 healthy individuals (33 male/27 female) were enrolled in this study. Patients were diagnosed according to WHO criteria [11]. Demographic details were obtained for all subjects; including age, sex, duration of diabetes, first-degree family history of diabetes. Controls had no personal or first-degree history of diabetes. Blood sampling was carried out from September 2012 to December 2012. One ml of venous blood sample was collected in EDTA tubes from each individual (patient or healthy control) and was stored as whole blood at −20 °C for subsequent DNA isolation.

All chemicals used in this study were of molecular biology analytical grade. Agarose was from peQ labs Co. PCR Master Mix (GoTaq® ready green Master Mix) was from Promega Co. and Thermo Co. Restriction enzymes (FastDigest® *HhaI*, and FastDigest® *RsaI*) were from *New Engl* and *BioLabs Inc*. Gel loading dye and DNA ladder (Gene Dire*X*® 100 and 50 bp ladder RTU ready to use) were from GeneDirex Co. Trizmabase, EDTA and borate were from Winlab Co. Ethidium bromide (EtBr) was from Biobasics Co. Isopropanol and ethanol were from Fluka.

In this study, the following five genes were genotyped (Fig. 1):

- *PTPN22*, [*SNP1858 C > T* (*rs#2476601*), *SNP-1123 C > G* (*rs#2488457*) and *SNP-2740 C ⩾ T* (*rs#1217412*)].
- *VDR* [*Fok1 SNP T > A* (*rs#2228570*) and *BsmI SNP-G > A* (*rs#1544410*)].
- *IL-10* [*SNP-1082 G > A* (*rs#1800896*) and *SNP-819 C > T* (*rs#3021097*].

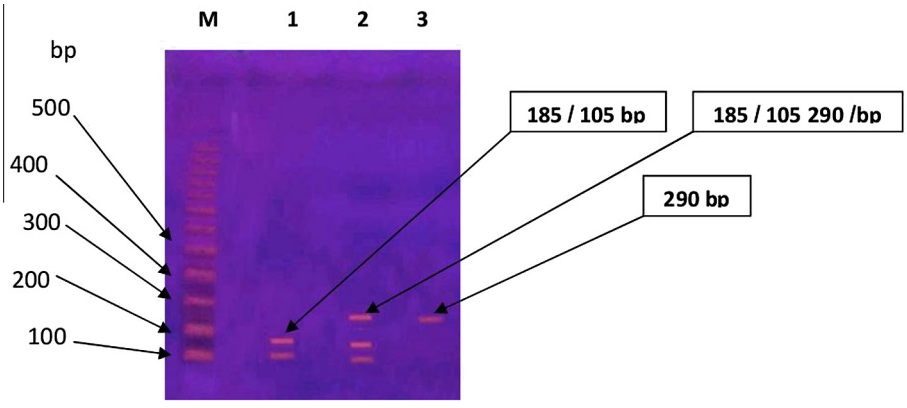

Figure 4 2% agarose gel electrophoresis for (RFLP) PCR for *VDR* SNP G > A (rs#1544410). M: 100 bp DNA ladder from GeneDire*X*®. Lane 1: PCR product upon using lane 1 represents homozygous GG genotype as the G allele PCR products cleaved by FastDigest® *HhaI* into 104 bp and 185 bp, lane 2 represents heterozygous GA genotype as G allele PCR products cleaved by Fast Digest® *RsaI* into 104 bp and 185 bp while A allele PCR products were still 290 bp, and lane 3 represents homozygous AA after cleaved by Fast Digest® *HhaI* still into 290 bp.

- *IL-18* [*SNP-607 C > A* (*rs#1946518*) and *SNP-137 G > C* (*rs#187238*)].
- *CTLA-4* [*SNP-49 G > A* (*rs#231775*)].

Genomic DNA was extracted using isopropanol precipitation as described by Sambrook et al. [12]. A partial fragment of the gene of interest containing the target SNP was retrieved from the dbSNP (an online single nucleotide polymorphism database). For allele specific PCR, four primers were designed, control forward, common reverse and two allele specific primers. The two allele specific primers for each SNP had the same nucleotide sequence except for the last nucleotide at the 3′ prime end of the primer.

Two molecular techniques were employed to amplify the target SNPs; allele specific PCR (Fig. 3) and Restriction Fragment Length Polymorphism-PCR (RFLP-PCR) (Fig. 4). *IL-18*, *IL-10*, and *CTLA-4* antigen *SNPs* were genotyped using allele specific PCR. PTPN22 and *Vit D receptor SNPs* were genotyped using both techniques.

Two restriction enzymes (RsaI and HhaI) were used in this study. The isolated DNA from all patients and controls under study was analyzed through agarose gel electrophoresis (Fig. 2).

3. Statistical analysis of data

Data were processed using SPSS package version 16. Descriptive statistics including frequency, distribution, mean, and standard deviation (SD) were used to describe different clinical characteristics. Univariate analyses including: *t*-test and Mann Whitney test were used to test the significance of results of quantitative variables and Chi-Square test was used to test for significance among categorized variables. Logistic regression was used to obtain odds ratios for variables with significant *P*-values. The confidence interval (CI) at 95% was used to describe the amount of uncertainty associated with the samples. The significance of the results was taken at the 5% level of significance.

The work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study protocol was approved by the review board of both the College of Medicine and the Institute of Graduate Studies and Research, University of Alexandria, Egypt and a written parental consent and child assent were obtained before the study.

4. Results

Table 1 shows that there was a significant difference in age between the two groups ($P = 0.0001$), to ensure that the controls were free from T1DM their mean age was 27.2 ± 6.4. There was no significant difference in sex between the two studied groups ($P = 0.173$). The disease onset range was 0.2–17 years with a mean of 5.3 ± 3.5 years.

The genotype distributions of *IL-18* in Table 2 did not significantly differ between T1DM patients and control subjects ($P = 0.641$) for the *SNP 607 C > A* (*rs#1946518*). On the other hand, there was a statistically significant difference between the two groups ($P = 0.001$) for the *SNP-137 G > C* (*rs#187238*) at the genotype level. Table 2 also shows that the genotypic distributions IL-10 did not significantly differ between the two groups, ($P = 0.208$) for the *SNP 819 C > T* (*rs#3021097*). On the other hand, there was a statistically significant difference between the two groups ($P < 0.0001$) for *the SNP 1082 G > A* (*rs#1800896*).

Table 2 shows that *SNP 49 G > A* (*rs#231775*) *in CTLA-4 gene* did not significantly differ between the patients and controls ($P = 0.341$).

For vitamin D-receptor polymorphism genotypic distributions; patients and controls did not significantly differ for the *SNP BsmI G > A* (*rs#1544410*), but there was a statistically significant difference between the two groups ($P = 0.004$) for the *SNP Fok1 T > A* (*rs#2228570*).

Table 2 also shows that three *SNPs* [*SNP-2740 C > T* (*rs#1217412*), *SNP-1123 C > G* (*rs#2488457*) and *SNP 1858 C > T* (*rs#2476601*)] in *PTPN22* gene were genotyped among the participant groups. It was revealed that the genotypic distributions did not significantly differ between the two groups ($P = 1.0$) for the *SNP-1858 C > T* (*rs#2476601*). On the other hand, there was a statistically significant difference between the two groups ($P < 0.000$) for the *SNP-2740 C > T* (*rs#1217412*) and the *SNP-1123 C > G* (*rs#2488457*).

A logistic regression model (Table 3) was adopted for significant results, only vitamin D-receptor *SNP Fok1 T > A* (*rs#2228570*), the *IL-18 SNP-137 G > C* (*rs#187238*)

Table 1 Age and sex among patients with T1DM diabetes and their controls.

Age and sex	Cases ($n = 60$)		Control ($n = 60$)		Significance	OR (95% CI)
	No.	%	No.	%		
Sex						
Male	25	41.7	33	55	$X^2 = 1.855$	–
Female	35	58.3	27	45	$P = 0.173$	1.65 (0.8–3.6)
Age (*years*)						
Min–Max	0.5–18.0	18.0–42.0	$Z = 9.474$			
Mean ± SD	11.2 ± 3.7	27.2 ± 6.4	$P = 0.0001^*$			
Disease onset (*years*)						
Min–Max	0.2–17.0	–				
Mean ± SD	5.3 ± 3.5	–				

X^2: Chi-Square; Z: Mann Whitney test; NA: Not applicable.
* Significant at $P \leqslant 0.05$.

Table 2 Studied polymorphic sites in some genes among patients with T1DM and their controls.

Gene polymorphism	Cases		Control		X^2	P
	No.	%	No.	%		
IL-18-607						
CC	11	18.3	15	25		
AA	10	16.7	8	13.3	0.89	0.641
CA	39	65	37	61.7		
IL-18-137						
GG	15	25	25	41.7		
CC	32	53.3	12	20	14.4	0.001*
GC	13	21.7	23	38.3		
IL-10-1082						
GG	17	28.3	8	13.3		
AA	22	36.7	4	6.7	26.3	0.0001*
GA	21	35	48	80		
IL-10-819						
TT	15	25	22	36.7		
CC	13	21.7	7	11.6	3.14	0.208
CTLA-4 49 G > A						
AA	6	10	2	3.3		
GG	9	15	10	16.7	2.149	0.341
AG	45	75	48	80		
Vitamin D-receptor BsmI SNP G > A						
GG	8	13.3	4	7.1		
AA	13	21.7	11	19.6	1.414	0.493
GA	39	65	41	73.2		
Vitamin D-receptor Fok1 T > A						
TT	16	26.7	7	11.7		
AA	21	35.0	12	20.0	33.7	0.000*
AT	23	38.3	41	68.3		
PTPN22 1858 C > T						
CC	1	1.9	0	0		
TT	12	22.2	12	22.6		$^{MC}P = 1.0$
CT	41	75.9	41	77.4		
PTPN22-1123 G > C						
GG	47	78.3	16	26.7		
CC	5	8.3	23	38.3	54.900	0.000*
GC	8	13.3	21	35		
PTPN22 + 274 A > G						
AA	36	60	20	33.3		
GG	10	16.7	14	23.3	19.600	0.000*
AG	14	23.3	26	43.3		

X^2: Chi-Square.
* Significant at $P \leqslant 0.05$.

Table 3 Logistic regression for *SNPs* with a significant *P*-value in patients and controls.

	B	S.E.	Wald	df	Sig.	Exp(*B*)	95.0% CI	
Il-10-1082	.443	.313	2.002	1	0.157	1.557	0.843	2.876
VDR.(Fok1)	.809	.372	4.737	1	0.030	2.245	1.084	4.650
IL-18-137	−.861-	.387	4.956	1	0.026	0.423	0.198	0.902
PTPN-1123	.940	.322	8.531	1	0.003	2.561	1.363	4.813
PTPN-2740	.199	.346	0.330	1	0.566	1.220	0.619	2.406
Constant	−3.280-	.921	12.689	1	0.000	0.038		

B = regression co-efficient; S.E. = slandered error; OR = adjusted odd ratio; CL = confidence level.

and the *SNP-1123 C > G* (*rs#2488457*) in PTPN22 *gene* showed the most significant difference between patients and controls.

5. Discussion

The etiology of human type 1 diabetes is still largely obscure, but it is recognized that both genetic and environmental factors are important in defining disease risk. A major focus of the current research is on the identification of putative risk genes with rarer or structural variants that could contribute to disease, and it is possible that the regions showing some evidence of linkage harbor variants that are not common *SNPs* well covered by the currently available genotyping platforms [8].

The aim of this study was to evaluate polymorphic sites in some genes associated with type 1 diabetes mellitus in a sample of Egyptian children.

The results showed, in *IL-18* gene, that there was a statistically significant difference between the patients and controls for the *SNP-137 G > C* (*rs# 187238*) at the genotype and allele levels. This result suggests that the genotype CC is a risk factor for T1DM, Novota et al. [13] in 2005 suggested that the two variants *SNP-607 C > A* (*rs#1946518*) and *SNP 137 G > C* (*rs#187238*) of *IL-18* gene are not associated with adult type 1 diabetes or latent autoimmune diabetes in adults (LADA) susceptibility. Conversely, Dong et al. [14] in 2007, found that the CC genotype at position-607 in the promoter region of the *IL-18* gene was significantly higher (risk factor) in Chinese Han children with T1DM than that in controls, while the AA genotype in -607 position could have a protective role for T1DM.

Present findings were partially in accordance with those of Mojtahedi et al. [15] in 2006 who found that there was no significant difference in the distribution of allele and genotype at positions -137 and -607 of *IL-18* gene between T1DM patients and control subjects without categorization of patients according to their age in Iranian population. On the other hand, the present findings regarding allele and genotype at position -137 of *IL-18* gene were in agreement with those of Massoud et al. [16] in 2009 in Iranian population and those of Kretowski et al. [17] in 2002 in Polish population. Both of them found that the frequency of GG and CC genotypes at position 137 may be associated with susceptibility to diabetes.

The genotype GA in *IL-10 SNP* 1082 G > A (rs#1800896) (Table 2) was found to be protective from the disease. Studies conducted in France and Spain did not confirm any significant association of T1DM with different genotypes of *IL-10* promoter polymorphisms, in Caucasians population. [18,19] Mohebbatikaljahi et al. [20] in 2009, showed no link between T1DM and *SNP-819* in *IL-10*, but previous studies in Turkish population showed that at the *IL-10 SNP-1082* (*A*/*G*) polymorphic site, the frequencies of GG genotype in patient and controls showed a significant difference. This genotype was more prevalent in the control group, thus, the G allele may be a protective allele and genotype 'GG' may have a protective effect against T1DM [20]. It has been proposed that variable production of Th2 cytokines including IL-10 may influence both the degree of β-cell destruction and the age of clinical onset [21].

In this study, the *CTLA-4* polymorphisms (*SNP 49 G > A rs#231775*), allele and genotypic distributions did not significantly differ between the patients and controls, Yanagawa et al. [22] in 1999, investigated the distribution of a *CTLA-4* gene polymorphism in Japanese patients with IDDM and control subjects, the study did not support an association between the *CTLA-4 gene* and IDDM, on the other hand Ma et al. [23] in 2002, in Han Chinese proved that *CTLA-4 49 AA* is protective from diabetes mellitus, whereas, *CTLA-4 49 G* allele (both as homozygotes and as heterozygotes) confers an increased risk of diabetes mellitus.

The *VDR* polymorphisms allele and genotypic distributions did not significantly differ in patients and controls for the *SNP BsmI G > A* (*rs#1544410*). On the other hand, there was a statistically significant difference between the two groups for the *SNP -Fok1T > A* (*rs#2228570*), this denotes that TA genotype may be a risk factor for T1DM in Egyptian children. In the U.K. populations, Nejentsev et al. [24] in 2004, indicated that common sequence variation in the *VDR* gene had no major effect in T1DM in both loci. Lemos et al. [25] in 2008, suggested that the single nucleotide polymorphisms of the *VDR* gene are unlikely to contribute significantly to T1DM susceptibility in both loci in the Portuguese population. On the other hand Wang et al. [26] in 2012, suggested that *FokI* polymorphism in the *VDR gene* is not associated with T1DM especially in East Asians, while *Bsm I* polymorphism in the *VDR* gene was associated with T1DM risk, especially in East Asians. The apparent discrepancies between studies could be a result of the effect of ethnic differences related to the distribution of *VDR* polymorphisms, as well as interactions with other genetic or environmental factors involved in the pathogenesis of type 1 diabetes mellitus.

For the three *PTPN22* polymorphisms (*SNP + 2740 C > T rs#1217412* and *SNP-1123 C > G rs#2488457* and *SNP 1858 C > T rs#2476601*), the distribution analysis of the allelic and genotypic frequencies revealed that there was no significant difference between patients and controls for the *SNP 1858 C > T* (*rs#2476601*). On the other hand, the difference for the *SNP + 2740 C > T* (*rs#1217412*) was statistically significant. The result showed that GG and CC genotype may be a risk factor for T1DM, in addition, there was a statistically significant difference between the two groups for the *SNP -1123 C > G* (*rs#2488457*) the result showed that AA genotype may be also a risk factor for T1DM in the Egyptian children.

These results disagree with Tang et al. [27] in 2012, who proved that T1DM is associated with *PTPN22 -1858C/T* gene polymorphism in Chinese population and targeting this promoter polymorphism should be dependent on ethnicity. Xuan et al. [28] in 2013, in ethnicity-and sex-stratified analyses, found similar associations among Caucasians and within Caucasian male and female strata. The meta-analysis results suggest that the *PTPN22 C1858T* polymorphism was associated with susceptibility to T1DM among the Caucasian population, and males who carried the *−1858T allele* were more susceptible to T1DM than females. Also Lavrikova et al. [29] in 2009, found that a given polymorphic marker was not statistically significant with T1DM in the transmission disequilibrium test, while analysis of the distribution of frequencies of alleles and genotypes showed the association with T1DM. Thus, the polymorphic marker *C1858T* of the *PTPN22* gene is associated with T1DM in Russian patients. On the other hand Santiago et al. [30] in 2007, proved that the *PTPN22 1858T* allele is a

T1DM susceptibility factor also in the Spanish population and it might play a different role in susceptibility to T1DM according to sex in early-onset T1DM patients.

Otherwise Cinek et al. [31] in 2007, proved that in two different Caucasian populations, the Czechs and the Azeri, no independent contribution can be detected either of the -1123 promoter SNP or the *+2740 3′-UTR SNP*, and only the minor allele at *PTPN22 codon 620* contributes to the risk of autoimmunity.

Liu et al. [32] in 2012, revealed that the *−1123 G > C* promoter polymorphism of *PTPN22* gene, but not the +1858 C > T variant, is associated with LADA in adult Chinese Hans. Tang et al., [27] in a systematic review and meta-analysis for (*−1858C/T*, *−1123G/C*) polymorphisms with type 1 diabetes mellitus detect no link for *PTPN22-1123G/C* polymorphism in Europe, America, and Asia. Kawasaki et al. [33] in 2006, proved that a regulatory *SNP-1123G/C* (*rs#2488457*) was shown to be weakly associated with T1DM in Japanese and Koreans.

To conclude, *IL-18 SNP-137 G > C* (*rs#187238*), *VDR SNP -Fok1 T > A* (*rs#2228570*), and the *SNP -1123 C > G* (*rs#2488457*) *in PTPN22* gene may have an effect on the occurrence of T1DM in Egyptian children. Further research including large-scale, population-based, case-control studies is needed.

Conflict of interest

The authors declare no conflict of interest. There is no financial and personal relationship with other people or organizations that could inappropriately influence this work.

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