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Genetic polymorphisms of Endoplasmic reticulum amino peptidase 1 (ERAP1) and Interferon lambda 4 (IFN-λ4) in Egyptian patients with type 1 diabetes mellitus

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Abstract

Different genetic and environmental factors are implicated in type I diabetes (T1DM) pathogenesis. About 50% of the genetic susceptibility for T1DM is related to human leukocyte antigen (HLA) genes. Other non-HLA genes have variable roles in the destruction of pancreatic β cells. A highly variable gene called endoplasmic reticulum associated with antigen processing gene 1(ERAP1) shares in activating autoreactive CD8+ T lymphocytes, peptide trimming, and subsequent pancreatic β cells destruction. Local production of inflammatory cytokines within the cells of islets of Langerhans is linked to T1DM progression. Different viral and autoimmune disorders have been linked to genetic variations in type III interferon (IFNλs). This study aimed to determine genetic polymorphisms of interferon lambda 4 (IFNλ4rs 73555604) and endoplasmic reticulum aminopeptidases 1 (ERAP1 rs26618) in Egyptian patients with T1DM. The study recruited 120 patients with T1DM from Kafrelsheikh University Hospital and 100 normal controls who were age and sex matched with the patients' group. Single-nucleotide polymorphism (SNP) genotyping of ERAP1(rs26618) and IFN-λ-4(rs73555604) was performed using real-time polymerase chain reaction. Patients with CC genotype were less likely to develop T1DM than those with TC and TT genotypes for both genes. In addition, T allele frequency in comparison to C allele frequency was significantly increased in T1DM patients when compared to control group (p<0.001). There were positive correlations between studied SNPs for both genes, fasting and postprandial blood glucose levels which suggest the association of these genes with T1DM occurrence. We concluded that the studied SNPs of ERAP1gene (rs26618) and IFNλ-4 gene(rs73555604) may be associated with T1DM development. In addition, T alleles for both genes could be considered risk alleles while C alleles would be regarded as a protective allele. Patients with TC and TT genotypes would be at a higher risk for T1DM than those carrying CC genotype.

Keywords: Type 1 diabetes (T1DM), IFN-λ4 gene, ERAP1 gene, polymorphisms.

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Introduction

Type 1 diabetes (T1DM) is an autoimmune, metabolic, and chronic disease that affects individuals who are genetically predisposed to disease development. T1DM is likely influenced by environmental factors such as medicines, chemicals, foods, and viruses. It is characterized by the loss of β cells of islets of Langerhans. [1] The signal peptide of preproinsulin epitopes, which is displayed by human leukocyte antigen (HLA) class I and activates CD8+T-cells, is primarily responsible for β -cell death in T1DM. [2]

According to genome-wide association studies (GWAS), human leukocyte antigen genes (HLA genes), in particular HLA class II loci, account for nearly 50% of the genetic susceptibility to T1DM. Other non- HLA genes have been known to be linked with the onset of the disease.[3] Some of these genes are related to antigen processing and presentation as endoplasmic reticulum associated with antigen processing gene (ERAP). [4]

Two isoforms of ERAP gene can be identified: ERAP1 and ERAP2. ERAP1 is a protein which belongs to a family of M1 zinc metallopeptidases, They are crucial in the process of trimming peptides so that they can be displayed by major histocompatibility complex (MHC) class I molecules, which activates autoreactive CD8+ T lymphocytes and then destruction of pancreatic β cells.[5]

Progression of T1DM is linked to local secretion of inflammatory cytokines within β cells of islets of Langerhans.[6] Among these cytokines' interferon family members.[7] Interferons (IFN) family includes 3 main subgroups: type I (IFNα, IFNβ, IFNω, IFNε, and IFNκ), type II (IFNγ), and type III (four IFNλ subtypes). [7] There are four different variants of IFN λ; IFNλ1 (IL-29), IFNλ2 (IL-28A), IFNλ3 (IL-28B) and IFNλ4 which are pseudogenes. Dinucleotide polymorphism in the IFN λ locus results in a frameshift mutation resulting in expressing functional IFNλ4 gene product.[8]

IFNλs can induce type I interferons mainly IFNα which is a major factor in the development of T1DM and they can also increase the synthesis of chemokines by human plasmacytoid dendretic cells (pDCs). They have the ability to increase HLA-I, HLA-II, and co-stimulatory molecules expression on pDCs, with subsequent activation of autoreactive CD8+T cells. [9] It is possible to define a set of single-nucleotide polymorphisms (SNPs) in the genes for IFN-ligand and receptor most importantly those that have been linked to various infectious and autoimmune diseases.[9] In addition IFNλs can induce type I interferon signaling molecules which is a key trigger of T1DM.[10]

The current study aimed to identify polymorphisms in the interferon lambda 4 (IFNλ4) and endoplasmic reticulum amino peptidase 1 (ERAP1) (rs26618) genes in Egyptian patients with T1DM.

Subjects and Methods

Our study enrolled 220 participants in two groups, Group 1 involved 120 T1DM patients and group II included 100 age and sex matched normal controls. Patients included in our study were recruited from Internal Medicine department, Kafrelsheikh University hospital.

The Ethical Committee of Medical Research Institute, Alexandria University, Egypt has revised and approved the study protocol (E/C S/N T49/2018: October 2018). All participants signed written informed consents. Full history taking, and clinical examination were done for all participants.

Sample collection

Venous blood samples (6 ml) were collected from each study participant. Of these, 3 ml were collected in EDTA tubes for hematological investigations and molecular assays. Other 2 ml were collected in plain test tubes for sera separation to be used in biochemical studies. Finally, 1ml was collected in sodium citrate tubes for erythrocyte sedimentation rate (ESR) assessment.

Hematological and biochemical investigations

Complete blood count (CBC), fasting blood glucose levels (FBG), post prandial blood glucose levels (PPBG), glycated hemoglobin levels (HbA1C), ESR, liver functions tests, and

renal functions tests were assessed for all subjects as described before. [11,12]

Detection of IFNλ-4 rs73555604 and ERAP rs26618 polymorphisms

DNA extraction

Genomic DNA was extracted from whole blood using QIAamp-spin columns kits (QIAamp DNA Blood Mini Kit, Applied Biosystems-Life Technologies), according to the manufacturer's instructions. [13] Then the extracted DNA samples were kept at -80° C until used.

SNP genotyping using real-time PCR

SNP genotyping of IFN λ4 (rs73555604) and ERAP1 (rs 26618) was carried out using the TagMan® SNP genotyping assay kit (stepOne real-time PCR, Applied Biosystems-Life Technologies, Carlsbad, California, USA),[14] Figures 1 and 2.

Each TagMan® SNP genotyping assay kit included sequence-specific primers, two allele-specific TagMan® probes to distinguish between both T and C alleles; VIC® fluorescent dye at the 5' end of the T allele's probe, and FAM (6-carboxyfluorescein) fluorescent dye at the 5' end of the C allele's probe, as well as a non-fluorescent quencher (NFQ) was linked to the 3' end of each probe.[14]

PCR amplification

PCR amplification was done using Step One real-time PCR system. All reagents were brought to room temperature (20-25℃) prior to use. In each PCR tube, 25 µl of reaction mix was prepared. Each mix contained 12.5 µl TaqMan Universal PCR master mix, 1.25 µl TaqMan SNP Genotyping Assay, 9.25 µl DNase-free water and 2 µl of the extracted DNA samples. The thermal cycling program conditions included: heating at 95℃ for 10 min., 40 cycles each of heating at 92℃ for 15 sec, at 60℃ for 1 min, then the reaction was hold at 4℃.

Interpretation of results

Expression of VIC dye only denotes homozygosity for the mutant allele (T/T), whereas an increase in FAM dye only denotes homozygosity for the wild allele (C/C). Increased fluorescence signals for both alleles (C/T) indicates heterozygosity. [15]

Statistical analysis

Statistical analysis of data was done using IBM SPSS software package version 20.0. Data were fed into a computer and evaluated, IBM Corp., Armonk, New York (Kirkpatrick and Feeney, 2013). The normality of the distribution was evaluated using the Kolmogorov-Smirnov test. Quantitative data were determined using the range, mean, and standard deviation. ANOVA was used to compare data from more than two groups, whereas the student t-test was employed to analyze data from the two study groups. To compare categorical variables within different groups, the chi square test was used. Odds ratio and confidence interval were then used to compare the risk group with the non-risk group. Hardy-Weinberg equilibrium (HWE) was employed to investigate possible deviations within the studied population. At the 5% level, significance of the results was determined.[16,17]

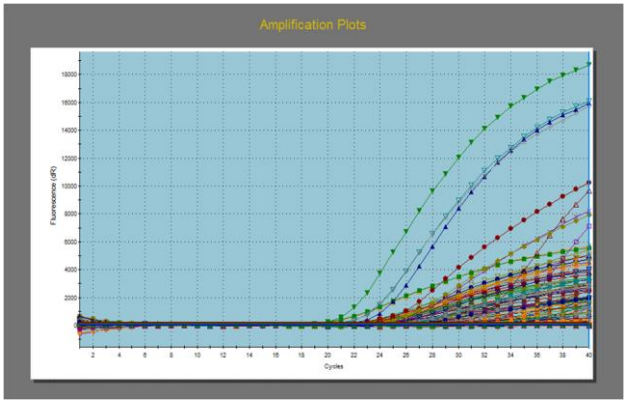

Figure 1. Amplification plots for ERAP (rs26618) SNP.

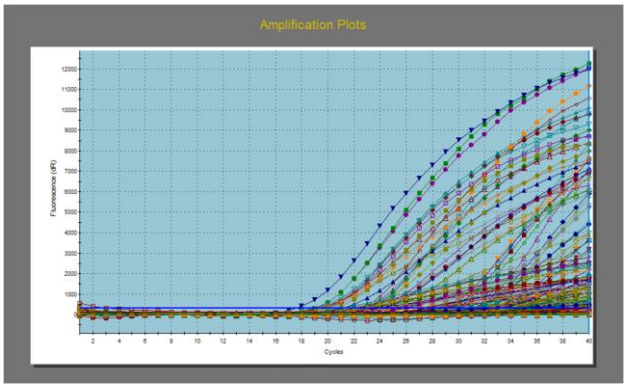

Figure 2. Amplification plots for IFN-λ-4 (rs73555604) SNP.

Results

Demographic data of study subjects

The mean age of T1DM patients ranged between 20.37 ± 2.25 years. About 56.7 % of the patients in the study were females compared to 43.3% of the male patients. No significant variation was observed between the studied groups regarding age and sex distribution (p=0.107, p=0.516, respectively). Of the patient's group, 16% had T1DM family history.

Genotype distribution and allelic frequency of ERAP1 (rs26618) and IFNλ-4 (rs73555604)

The HWE revealed no deviation of the studied genotypes within all participants (p>0.05). SNP genotyping of both genes was determined using 5´ nuclease allelic discrimination assay (TaqMan SNP genotyping assay). In T1DM patients TC genotype for both ERAP1(rs26618) and IFNλ-4 (rs73555604) genes was the most prevalent among T1DM patients while CC genotype for both ERAP1 (rs26618) and IFNλ-4 (rs73555604) genes was the commonest among the control group.

Patients with TC and TT genotypes for both ERAP1(rs26618) and IFNλ-4 (rs73555604) genes were at higher risk for T1DM development than those carrying CC allele (OR: 12.017, 9.205) (CI :5.225-27.639, 3.182-26.625) for ERAP1 gene, and (OR: 21.190, 20.417) (CI 6.197-72.465, 5.403-77.156) for IFNλ-4 gene.

For both studied SNPs a significant statistical difference was found in T allele frequency (mutant allele) when compared to C allele frequency (wild allele) among studied groups (p<0.001), Table 1.

Table 1. Genotyping distribution and allelic frequency of ERAP1 (rs26618) and IFNλ-4 (rs73555604) in studied groups.

	T1DM patients (n =120)		Control group (n =100)		≠* p value	OR	95% CI (LL-UL)
ERAP1 (rs26618)	No.	%	No.	%			
CC®	8	6.7	45	45.0	<0.001		
TC	94	78.3	44	44.0	<0.001	12.017	5.225-27.639
TT	18	15.0	11	11.0	NS	9.205	3.182-26.625
Allele							
C®	110	45.8	134	67.0	<0.001		
T	130	54.2	66	33.0		2.399	1.626-3.541
IFNλ-4 (rs73555604)							
CC®	3	2.5	35	35.0	<0.001		
TC	89	74.2	49	49.0	<0.001	21.190	6.197-72.465
TT	28	23.3	16	16.0	NS	20.417	5.403-77.156
Allele							
C®	95	39.6	119	59.5	<0.001		
T	145	60.4	81	40.5		2.242	1.529-3.289

≠χ^2: Chi square test; MC: Monte Carlo; OR: Odds ratio CI: Confidence interval.
LL: Lower limit; UL: Upper Limit; * P > 0.05 is not significant (NS).

Correlation analysis

IFN-4 (rs73555604) gene exhibited a significant positive correlation solely with FBG and PPBG levels, (p<0.008, p<0.023, respectively) whereas ERAP1 gene showed a significant positive correlation with RBCs count as well as FBG, and PPBG levels (p= 0.046, p<0.001, p<0.010, respectively) (Table 2).

Table 2. Correlation between ERAP1 (rs26618) and INF (rs73555604) with different parameters in the study 120 T1DM patients.

Studied parameters	ERAP1 (rs26618)		INF (rs73555604)	
	r_s	*p value	r_s	p value
Hb	0.085	NS	0.072	NS
RBCs	0.183	0.046	0.099	NS
WBCs	0.137	NS	0.165	NS
Platelet	-0.027	NS	-0.130	NS
FBS	0.374	<0.001	0.241	0.008
PPBG	0.234	0.010	0.208	0.023
HBA1C	0.127	NS	0.139	NS
ESR	-0.028	NS	-0.130	NS
RBS	-0.006	NS	-0.020	NS

r_s: Spearman coefficient; **P* > 0.05 is not significant (NS).

Hematological investigations

Both hemoglobin (Hb) levels and red blood cells (RBCs) counts showed a significant reduction in T1DM patients in comparison to control group (p<0.001). Regarding white blood cells (WBCs) counts no significant statistical difference was detected between both groups (p=0.925). Statistical analysis of platelets count revealed a significant increase in platelets count among T1DM patients when compared with the controls (p<0.001). In addition, ESR levels were significantly high in the T1DM patient group (p<0.001), (Table 3).

Blood glucose profile

Blood glucose profile revealed that FBG, random blood sugar (RBG), PPBG, and HbA1C levels of T1DM patients were markedly higher than controls (p <0.001) (Table 4).

Biochemical investigations

A slight elevation in liver enzymes (SGOT and SGPT) and renal function tests (serum creatinine and urea) was observed in T1DM patients in respect to controls (Table 5).

Table 3. Comparison between the two studied groups according to hematological findings.

Hematological parameters	T1DM patients (n =120)	Control group (n =100)	*p value
Hb (Mean ± SD)	12.61 ± 1.27	14.49 ± 0.63	<0.001
RBCs (Mean ± SD)	4.41 ± 0.59	5.25 ± 0.83	<0.001
WBCs (Mean ± SD)	6.89 ± 1.51	6.88 ± 1.50	NS
Platelets (Mean ± SD)	236.08 ± 92.24	166.82 ± 12.57	<0.001
ESR (Mean ± SD)	11.51 ± 3.14	9.25 ± 1.68	<0.001

t: Student t-test for comparing between the studied groups. *P* > 0.05 is not significant (NS).

Table 4. Comparison between the studied groups according to diabetic profile.

Diabetic parameters	T1DM patients (n =120)	Control group (n =100)	*p value
FBG (Mean ± SD)	223.85 ± 33.10	77.27 ± 4.14	<0.001*
PPBG (Mean ± SD)	360.25 ± 70.14	83.93 ± 4.83	<0.001
RBG (Mean ± SD)	360.26 ± 48.28	83.35 ± 3.29	<0.001
HBA1C (Mean ± SD)	7.91 ± 0.48	4.29 ± 0.27	<0.001

t: Student t-test for comparing between the studied groups. **P* ≤ 0.05 is significant.

Table 5. Comparison between the studied groups according to biochemical investigations (liver and renal function tests).

Biochemical parameters	T1DM patients (n =120)	Control group (n =100)	*p value
S GPT (Mean ± SD)	19.14 ± 1.62	18.23 ± 1.97	<0.001
S GOT (Mean ± SD)	21.56 ± 2.02	21.89 ± 3.20	NS
Serum creatinine (Mean ± SD)	0.84 ± 0.17	0.79 ± 0.08	0.005
Urea (Mean ± SD)	23.46 ± 2.67	23.38 ± 2.27	NS

t: Student t-test for comparing between the studied groups. *P* > 0.05 is not significant (NS).

Discussion

This study aimed to determine IFNλ4 (rs 73555604) and ERAP1 polymorphisms (rs26618) in Egyptian patients with autoimmune T1DM. To achieve this goal this study was carried out on 120 patients with T1DM and 100 normal controls who were age and sex matched with the patients group. We evaluated the genotype distribution and allele frequency of the ERAP1 gene to detect the relationship between this gene and the risk of T1DM occurrence. According to our findings, T allele frequency (mutant allele) increased much more frequently in T1DM patients than in the control group (p<0.001) compared to C allele frequency (wild allele). Additionally, the TC genotype was the most prevalent in the patients group. In concordance to our findings, Reeves et al., 2018, revealed a link between the development of T1DM and several ERAP1 haplotypes.[4] A different study by Fierabracci et al., 2012, revealed that allelic variations of ERAP1 gene were reported in a variety of chronic inflammatory disorders including multiple sclerosis, T1DM, Crohn's disease and Behçet's disease.[18]

TC and TT genotypes showed variable distribution between the studied groups in comparison to CC genotype (p=0.001, p=0.383, respectively). These results indicate that T allele, TC and TT genotypes may be linked to the risk of T1DM development, whereas C allele could be regarded as a protective allele and that CC genotype, more prevalent in the control group, could possibly be linked to a lower risk of disease development.

In contrast to our findings Gianchecchi et al., 2013, revealed that ERAP1 gene played a minor role in the pathogenesis of T1DM in the Italian population because there was no significant difference in allelic frequencies between T1DM patients and the control group as they were distributed equally in both groups. This can be explained by the phenomenon that different ethnic groups will carry alleles with different frequencies and genetic distributions.[19]

Up to our knowledge this is the first study to determine IFN-λ-4 genotypes in T1DM. Genotype distribution and allelic frequencies of IFN-λ-4 were identical to those of the ERAP1 gene. Patients with T1DM showed a significantly higher frequency of the mutant T allele than the wild C allele than the controls (p<0.001). In comparison to CC allele, TC and TT genotypes showed distinct distributions in T1DM patients (p=0.001, p=0.176, respectively). From the above results we can suggest that patients with TC and TT genotypes may be at higher risk to T1DM than those carrying CC genotype.

In line with our results Jean-Baptiste et al., 2017, clarified that IFNλs can increase type I interferon which is a key trigger of T1DM along with increased production of inflammatory cytokines and chemokines within β cells of islets of Langerhans.[20] In addition Domsgen et al., 2016, revealed changes of the IFN signature in pancreatic β cells of islets of Langerhans which is induced by IFNλs following *in vitro* infection with Coxsackievirus B3.[21]

Regarding demographic data, the control group's mean age was 20.86 ±2.26 years, whereas the patient group's mean age was 20.37 ±2.25 years. No difference was noticed between the two groups (p=0.107). Regarding gender distribution, females represented 56.7% of studied patients and 61% of the control group while males represented 52% of studied patients and 39% of the control group. No statistically significant difference was found between both groups (p=0.516). Results of

demographic data in the present study were not in concordance with other studies carried out by Mohamed et al., 2022[22] and Assmann et al., 2017.[23] Gender difference in T1DM incidence can be attributed to variability in the socioeconomic environment. A recent study carried out by Tatti et al., 2022, supported our findings and reported that gender difference may be due to hormonal factors.[24]

Most of the studied patients had no family history of T1DM and this finding contrasted with that of Krischer et al., 2017, who found that incidence of T1DM was increased in individuals with positive family history.[25] However, Cernea et al., 2010, reported that only 10-15% of the studied patients had a positive family history and this finding was in accordance with our study. [26]

Concerning hematological parameters, our findings demonstrated that T1DM patients had a significantly lower Hb levels and RBCs counts than the control group. These findings are consistent with those of a previous study by Thomas et al., 2002, who reported the occurrence of anemia in patients with T1DM due to the failure of the kidney to increase erythropoietin production in response to decreased Hb levels.[27] In this study, WBCs count showed no significant variation between both groups and this finding was not in accordance with a study carried out by Abdel Moneim et al., 2020, who observed, increased WBCs and neutrophil counts in T1DM patients as a result of autoinflammation and autoimmunity.[28] Furthermore, a significant increase in platelets count was observed in our patient's group in comparison to the control group. The later finding contrasted with those of Abdel Moneim et al., 2020, who observed decreased platelets count in T1DM patients.[28]

ESR is known as an inflammatory marker which is associated with autoimmunity and autoinflammation. In the current study, T1DM patients showed higher ESR levels when compared to the control group ($p<0.001$). In line with our results Guo et al., 2020, concluded that diabetic patients especially those with diabetic ketoacidosis have a higher ESR levels in comparison to their control group. [29]

As expected, we observed that T1DM patients exhibited higher levels of RBG, FBG, PPBG, and HBA1C %. These findings are in agreement with those of Kahanovitz et al., 2017, who found higher levels of RBS, FBS, two-hour plasma glucose, and HbA1C % in diabetic patients.[30] Concerning liver enzymes, we observed a significant increase in AST levels in T1DM patients in comparison to the control group ($p<0.001$). However, ALT levels showed no significant variation between both groups ($p<0.371$). Additionally, our findings of renal function tests showed no difference in urea levels while T1DM patients' creatinine levels were significantly higher than the control group ($p<0.821$; $p<0.005$, respectively).

According to Adiga et al., 2016, who reported elevated liver enzymes and low glomerular filtration rate in diabetic patients, the results of liver and renal function tests of our study were in consistent with those findings, and they may serve as an indicator for the onset of diabetic hepatopathy and nephropathy.[31]

Correlation analysis revealed positive association between both studied genetic polymorphisms and other blood glucose indices (FBG and PPBG) levels. These findings can suggest an association between ERAP1 gene (rs26618), IFNλ4 gene (rs73555604) and elevated blood sugar levels in Egyptian patients with autoimmune T1DM.

From our results we can conclude that the studied SNPs of ERAP1gene (rs 26618) and IFNλ-4 gene (rs 73555604) may be associated with T1DM development. In addition, T allele for both genes could be considered as a risk allele while C allele regarded as a protective allele. Patients with TC and TT genotypes might be at a higher risk for development of T1DM than those carrying CC genotype.

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Author Contributions

SAE, AAG and EMO; contributed to the study conception and design. AAG, EMO and MN; contributed to material preparation, data collection

and analysis. IA and ST; provided clinical support. EMO; wrote the manuscript draft. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Ethics Committee of the Medical Research Institute, Alexandria University (E/C. S/N. T49/2018).

Informed consent

Before enrollment in the study, all participants provided written informed consent for their involvement in the clinical research study.

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