

Association of MiRNA-21 (rs1292037) and CTLA-4 (1822 C/T) Gene Polymorphisms with Type 1 Diabetes Mellitus in Iraqi Children and Adolescents

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ABSTRACT

Background: Type 1 diabetes mellitus (T1DM) is a prevalent chronic autoimmune disease affecting children and adolescents, characterized by immune-mediated destruction of pancreatic β -cells, leading to insulin deficiency. Genetic susceptibility plays a crucial role in disease development, with single nucleotide polymorphisms (SNPs) in immune-related genes, such as CTLA-4 and miR-21, potentially influencing disease risk. **Objective:** This study aimed to evaluate the association between miR-21 (rs1292037) and CTLA-4 (1822 C/T) gene polymorphisms and the susceptibility to T1DM in Iraqi children and adolescents. **Methods:** A case-control study was conducted involving 100 pediatric patients diagnosed with T1DM and 100 age-matched healthy controls. The study was carried out between September 2023 and December 2024. Genotyping of miR-21 (rs1292037) and CTLA-4 (1822 C/T) polymorphisms was performed using polymerase chain reaction (PCR)-based methods. **Results:** The miR-21 (rs1292037) polymorphism was significantly associated with T1DM, suggesting its potential role in disease pathogenesis ($p < 0.05$). However, the CTLA-4 (1822 C/T) polymorphism did not show a significant correlation with T1DM risk, indicating that it may not contribute to disease susceptibility in the studied population. **Conclusion:** The findings suggest that miR-21 (rs1292037) could serve as a potential predictive and diagnostic biomarker for T1DM, while CTLA-4 (1822 C/T) may not be a major genetic determinant in the studied Iraqi cohort. Further research with larger sample sizes and functional studies is recommended to elucidate the molecular mechanisms linking miR-21 to T1DM pathogenesis.

Keywords: Type 1 Diabetes, CTLA4, MiRna 21.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) has long-term impacts on the patient, their family, and society at large. Insulin insufficiency results from the selective death of pancreatic β cells in type 1 diabetes. T1DM may have a serious negative effect on the kidneys, heart, and nerves, among other organs. There is a lot of evidence to suggest that genetic factors are important for the development and pathogenicity of type 1 diabetes [1]. Concerns have been raised by epidemiological research on pediatric T1DM because of its rising global frequency. An estimated 0.3% of children in the US get a T1DM diagnosis each year [2]. Unlike high-income nations, the prevalence of this illness is still rising quickly in low- and middle-income countries. According to estimates from 2019, the illness killed 1.5 million individuals directly and 2.2 million people as a result of hyperglycemia [3]. The many manifestations of Type 1 Diabetes Mellitus (T1DM) arise from a blend of hereditary and environmental influences. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a particular factor that has a high affinity for binding with the B7 molecule on antigen-presenting cells. The binding of this substance results in a reduction in the activity of T-cells, hence halting the immunological response. This mechanism is crucial in determining an individual's susceptibility to T1DM [4].

The most common and significant kind of genetic variation are single-nucleotide polymorphisms (SNPs), which have been extensively researched and have the potential to have negative phenotypic effects. Unfathomably intricate theories suggest that SNPs in the miRNA coding regions may modify the target mRNA in addition to affecting the regulatory function and binding affinity of miRNAs. Numerous disorders, including type 1 diabetes, have been linked to the occurrence

of miRNA-related SNPs [5]. The worldwide incidence of diabetes has significantly risen since 1980. According to the World Health Organization, the number of adults living with diabetes was estimated to be 108 million in 1980, but by 2014, this number had increased to 422 million. The prevalence of diabetes among adults has increased from 4.7% to 8.5%, with a noticeable rise observed in all regions [6].

The incidence of Type 1 Diabetes Mellitus (T1DM) in Basra City ranges from 5 to 9.99 per 100,000 each year and saw a rise between the years 2012 to 2016. Likewise, in Al-Nasiriyah City, the incidence of the disease has also been on an upward surge in the most recent five-year period [7]. A perplexing characteristic of Type 1 Diabetes Mellitus (T1DM) is that, despite dysfunctional conditions affecting both pancreatic β -cells and α -cells, only the β -cells are destroyed by the immune system, while the neighboring α -cells are spared. This dysfunction culminates in death solely for β -cells. Recent discoveries indicate notable discrepancies between these cell types.

Firstly, the BCL2L1 gene, known for its anti-apoptotic function, has a higher expression in α -cells than in β -cells. Secondly, genes associated with endoplasmic reticulum (ER) stress show distinct expression levels. Pro-apoptotic CHOP expression is higher in β -cells while α -cells express higher levels of HSPA5 (encoding protective chaperone BiP). Thirdly, viral recognition and innate immune response genes have higher expression in α -cells, rendering them more resilient to coxsackievirus infection. Lastly, α -cells portray a higher expression of the immune-inhibitory HLA-E molecule, compared to β -cells [8]. The pivotal role of insulin as an autoantigen

is substantiated by the strong impact its gene deletion has on the progression of the disease. Administering insulin as a pre-diabetic phase or stage could potentially forestall or postpone the clinical symptoms of T1DM [9].

The key genes contributing to T1DM susceptibility reside as apart the major histocompatibility complex (MHC) area, commonly referred to as it is located on chromosome 6 and is called HLA (human leukocyte antigen). There is a 40%-50% genetic risk of developing T1DM associated with polymorphic alleles of the HLA complex. Genetic susceptibility linked to Susceptibility and protection associated with HLA class II, along with a polymorphism within the HLA area in MHC, predominantly HLA-DR and HLA-DQ are the most important loci to identify inherited risk for developing T1DM [10].

Polymorphisms within the CTLA-4 gene have been associated with Type 1 Diabetes Mellitus (T1DM) as well as other autoimmune conditions, these genetic variations lead to a decrease in the intracellular levels of the CTLA-4 protein, Consequently, the regulation of excessive activation and expansion of T lymphocytes becomes compromised, causing an unchecked advancement of the immune response and a disruption in autoimmune homeostasis [11]. Epigenetic regulation serves as a bridge between genetic information and environmental impacts, resulting in the observable phenotype of cells. In the context of T1DM, this factor plays a substantial GWAS studies identify numerous SNP variants mapped to enhancer regions, which are essential for transcription factor binding and, consequently, the regulation of gene expression [12]. The presence of single nucleotide polymorphisms (SNPs) in the genes responsible for coding these miRNAs may have an effect on pancreatic development and the production of insulin. Therefore, there is a correlation between the amount of miR-21 expression and pediatric T1DM, since there is a significant difference in the expression of the miR-21 gene between the patient and control groups with T1DM [13].

MATERIAL AND METHOD

Study Design

The case-control study was conducted in the Al-Najaf governorate. The participants included in this study were 100 patients (55 males and 45 females), all of whom were diagnosed with T1DM and aged between 3 and 18 years. The study also included a healthy control group of 100 individuals (55 males and 45 females), also ranging from 3 to 18 years in age. They were attended the consultant clinic for Diabetes and Endocrinology Center in Al-Sadr teaching hospital in the period from 10 September 2023 to 20 December 2024 under the supervision of medicine physician. Patients were diagnosed with diabetic T1DM according to the history, laboratory examination (HbA1c test). The data concerning patients were gathered from their personal records and direct patient-questionnaire, which included information such as patient number, family medical history and the treatment they're undergoing. Every participant gave their informed consent verbally. four milliliters of venous blood were drawn by disposable syringe under aseptic technique.

Ethical Approval

Before beginning sample collection for this study, the project received preliminary permission. Furthermore, the Scientific Research

Methodology Ethics Committee of Al-Qadisiyah University's Medicine College, Microbiology Branch approved the study's design. by the document numbered 30/5121 in the date of 10 September, 2023.

Laboratory Work

Two ml of blood were collected in ethylenediaminetetraacetic acid (EDTA) tube and stored at -20°C for DNA extraction for detection of *CTLA-4* 1822 C/T and *Mirna21* rs1292037 SNP gene polymorphism by Tetra-ARMS PCR.

Statistical Analysis

The data was collected, organised, analysed, and presented using Microsoft Office Excel 2019 and the statistical software SPSS version 20. Following the identification of normally distributed and non-normally distributed variables and conducting the Kolmogorov-Smirnov normality test, the numerical data were reported in terms of the mean and standard deviation.

The independent sample t-test was used to investigate the mean differences between any two groups, assuming that the variable followed a normal distribution. The chi-square test may be used to examine the connection between any two categorical variables. The risk was quantified by calculating the odds ratio and determining the 95% confidence interval. At a P-value of less than 0.05, the level of significance was deemed to be reached, and at a 0.01 or less, the very significant level.

Results

Analysis of the Gene Polymorphism

Detection of (*CTLA-4*) (1822 C/T) Gene Polymorphisms in Type 1 Diabetes Mellitus

Figure 1 displays an agarose gel electrophoresis picture illustrating the examination of the Tetra-ARMS-PCR product for the gene polymorphism (*CTLA-4* 1822 C/T) in both patients and healthy controls. The marker M ranges from 3000 to 100 base pairs. The lane (TT) wild type homozygote exhibited solely the T allele in the 162bp Tetra-ARMS-PCR product. The homozygote mutant type lane (CC) exhibited only the C allele in the 117bp T-ARMS-PCR product, whereas the heterozygote (TC) displayed both the T and C alleles in the 162bp and 117bp Tetra-ARMS-PCR product.

The external internal control was detected in the 326bp Tetra-ARMS-PCR product. The distribution of genotypes exhibited no divergence from the Hardy-Weinberg equilibrium. Hardy Weinberg equation was applied to (*CTLA-4*) (1822 C/T) genotypes, TT, TC and CC, distribution within the control group and results are shown in table (1). The homozygous wild genotype TT was seen in 73 out of 100 control participants, whereas the heterozygous TC genotype was observed in 25 out of 100 control patients.

The homozygous mutant CC genotype was observed in 2 out of 100 control subjects. The distribution of genotypes for (*CTLA-4*) (1822 C/T) in the control participants was found to be substantially different from what was predicted ($P = 0.001$). The table (2) presents the comparison of genotypes and allele frequencies of the (*CTLA-4*) (1822 C/T) SNP between individuals with T1DM and a group of healthy individuals.

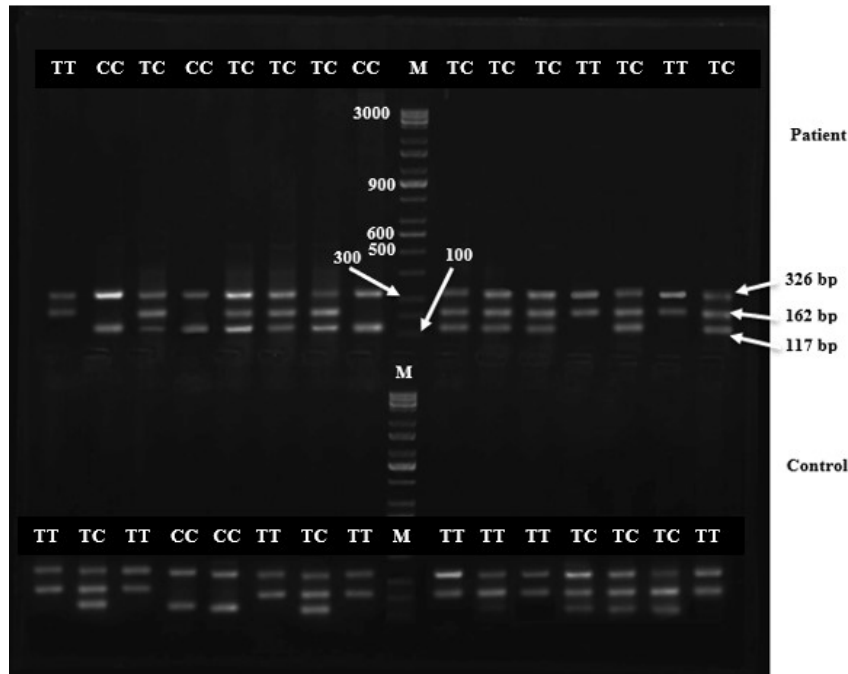


FIGURE 1: The Image of Agarose Gel Electrophoresis Presents the Tetra-ARMS-PCR Product Analysis of CTLA-4 1822 (C/T) Gene Polymorphism in Patient Samples. In This Case, M Refers to the Marker Ranging from 3000-100bp. The TT Wild Type Homozygote was Observed in the T Allele Exclusively, While the CC Mutant Type Homozygote was Observed Only in the C Allele. In Contrast, The T/C Heterozygote was Observed in Both T and C Alleles. The Existence of the T Or C Allele Was Observed at a 326 Bp Product Size.

Table 1: Hardy Weinberg Equation.

Genotypes	Observed	Expected	P
Homozygote Reference TT	19	52.5	0.001
Heterozygote TC	70	47.5	¥
Homozygote Variant CC	11	0.0	S

N: Number of Cases; SD: Standard Deviation; ¥: Chi-Square Test; S: Significant at $P < 0.001$

Table 2: (CTLA-4) (1822 C/T) Genotype Frequency in Patients and Healthy Control.

Mode	(CTLA-4) (1822 C/T)	Patients N= 100	Control N= 100	P	OR	95% CI	PF	EF
Co-Dominant	TT	19 (19%)	73 (73%)	0.001		Reference	-----	
	T/C	70 (70%)	25 (25%)	¥	0.29	0.19-0.43	----	2.49
	CC	11(11%)	2 (2 %)	S	3.15	2.2-4.5	----	0.63
Dominant	TT+T/C	89 (89%)	98(98 %)	0.001		Reference	-----	
	CC	11 (11%)	0 (2 %)	¥ S	0.89	0.83-0.95	----	0.12
Recessive	TT	19 (19%)	73 (73 %)	0.001		Reference	-----	
	T/C+CC	81 (81%)	27 (27 %)	¥ S	3.24	2.28-4.61	----	0.69
Alleles	T	108 (54%)	175 (87.5 %)	0.001		Reference	0.38	----
	C	92 (46%)	25 (12.5 %)	¥ S	3.68	2.48-5.47	----	0.73

N: Number Of Cases; SD: Standard Deviation; ¥: Chi-Square Test; S: Significant At $P < 0.001$

Detection of *miR-21gene (rs1292037)* SNP Gene Polymorphisms in Type 1 Diabetes Mellitus

Figure 2 displays an agarose gel electrophoresis picture illustrating the examination of the T-ARMS-PCR product for the *miR-21 gene (rs1292037)* SNP gene polymorphism. The marker M has a size range of 2000-100 base pairs. The lane (TT) wild type homozygote exhibited solely the T allele in the 231bp Tetra-ARMS-PCR product. The homozygote mutant type lane (CC) exhibited just the C allele at

184bp in the T-ARMS-PCR product. On the other hand, the heterozygote (TC) displayed both the T and C alleles at 231bp and 184bp in the Tetra-ARMS-PCR product. The external internal control was detected in the 364bp Tetra-ARMS-PCR product. The distribution of genotypes did not deviate from the Hardy-Weinberg equilibrium. Hardy Weinberg equation was applied to *miR-21gene (rs1292037)* SNP genotypes, TT, TC and CC, Within the control group are reflected in table (3).

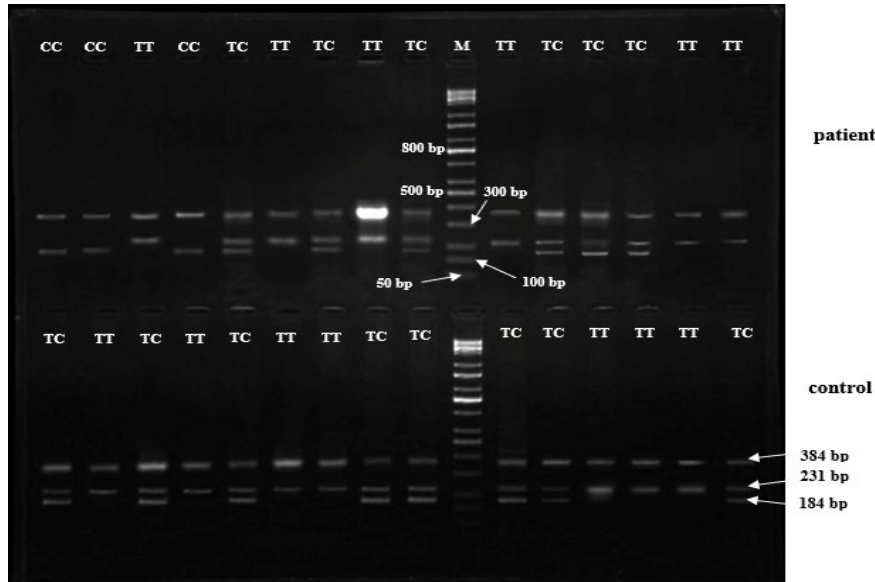


Figure 2: The Agarose Gel Electrophoresis Image Exhibits the Tetra-ARMS-PCR Product Analysis of Mir-21 (Rs1292037) SNP Gene Polymorphism in the Samples of the Patients. Here, M Represents the Marker with a Range from 800-50bp. The TT Wild Type Homozygote was Depicted Solely in the T Allele, while the CC Mutant Type Homozygote was Depicted Solely in the C Allele. Conversely, The T/C Heterozygote was Apparent in both the T and C Alleles. The Occurrence of Either T or C Alleles was Discerned at a 384 Bp Product Size.

Table 3: Hardy Weinberg equation.

Genotypes	Observed	Expected	P
Homozygote Reference TT	75	2.4	0.006
Heterozygote TC	25	4.5	¥
Homozygote Variant CC	0	1.1	S

N: Number Of Cases; SD: Standard Deviation; ¥: Chi-Square Test; S: Significant at $P < 0.001$

Out of the 100 patients in the control group, 75 had the homozygous wild genotype TT, and 25 had the heterozygous TC genotype. The homozygous mutant CC genotype, on the other hand, was not observed in any of the control subjects, as per table (4). The actual distribution of control subjects based on the miR-21gene (rs1292037) SNP genotypes markedly varied from the predicted distribution ($P = 0.006$).

Association between Family History of Patients with Type1 Diabetic Mellitus and the Results of Gene Polymorphism by T-ARMS-PCR

Association between family history of patients with T1DM and the results of T-ARMS-PCR *CTLA-4* 1822 C/T and *miR-21*gene (rs1292037) polymorphism was shown in table (5) and (6).

Table 4: The type1 Diabetic Mellitus Genotype Frequency in Patients and Healthy Control.

Mode	Mir-21gene (Rs1292037)	Patients N = 100	Control N = 100	P	OR	95% CI	PF	EF
Co-dominant	TT	17 (17.24%)	75 (75%)	0.001	Reference	-----		
	T/C	66 (65.52%)	25 (25%)	¥	0.8	0.71 - 0.89	----	0.25
	CC	17 (17.24 %)	0 (0%)	S	---	---	---	0.83
Dominant	TT	17 (17.0 %)	75 (75.0%)	0.001	Reference	-----		
	CC+T/C	83 (83.0 %)	25 (25.0%)	¥	3.32	2.34 - 4.72	----	0.7
				S				
Recessive	CC	17 (17.0%)	0 (0%)	0.001	-	--	---	---
	T/C+TT	83 (83.0%)	100 (100%)	¥	Reference	-----		
				S				
Alleles	C	100 (50.0%)	25 (12.5 %)	0.001	4	2.7 - 5.92	---	0.75
	T	100 (50.0%)	175 (87.5 %)	¥	Reference		0.43	---
				S				

N: Number of Cases; SD: Standard Deviation; ¥: Chi-Square Test; S: Significant At $P < 0.001$.

Table 5: Association between Family History of Patients with Type 1 Diabetic Mellites and the Results of Gene Polymorphism (*CTLA-4*) (1822 C/T) Gene Polymorphism).

Ctla-4		Family History		*P-Value	**Or
Genotypes		Positive	Negative		
	TT	3 (15.8%)	16 (84.2%)	0.34	wild
	TC	23 (32.9%)	47 (67.1%)	¥	1.04(0.87-1.24)
Allele	CC	3 (27.3%)	8 (72.7%)	NS	0.79(0.23-2.75)
	T	29 (26.9%)	79 (73.1%)	0.28	0.9(0.67-1.21)
	C	29 (31.5%)	63 (68.5%)	¥	1.13(0.82-1.55)
				NS	

¥: Chi-Square Test; OR: Odds Ratio; NS: Not Significant at $P > 0.05$; *: Significant at $P \leq 0.05$

Table 6: Association between Family History of Patients with Type1 Diabetic Mellites and the Results of Gene Polymorphism *Mir-21* gene (*rs1292037*) Polymorphism.

Mir-21		Family History		*P-Value	**OR
Genotypes	TT	Positive 5 (29.4%)	Negative 12 (70.6%)	0.91	wild
	TC	20 (30.3%)	46 (69.7%)	¥	0.95 (0.75-1.22)
	CC	6 (35.3%)	11 (64.7%)	NS	1.2 (0.5-2.88)
	T	30 (30%)	70 (70%)	0.44	0.95 (0.7-1.3)
Allele	C	32 (32%)	68 (68%)	¥	1.05 (0.78-1.41)
				NS	

¥: Chi-Square Test; OR: Odds Ratio; NS: Not Significant at $P > 0.05$; *: Significant at $P \leq 0.05$ **Table 7:** The Synergistic Effect of Multiple Risk Alleles in Association with Type1 Diabetic Mellitus (0 or 1 Risk Alleles Versus 2, 3 Or 4 Alleles).

Risk Alleles	Patients N = 100		Control N = 100		P	OR	95% CI
	N	%	N	%			
0 or 1	29	27.6	76	72.4	0.0001¥ S	Reference	
2	40	65.6	21	34.4		2.68	1.74 -4.11
3	25	89.3	3	10.7	0.0001 ¥ S	12.19	3.87 -38.37
4	6	100.0	0	0.0	0.001 ¥ S	---	---

N: Number Of Alleles; ¥: Chi-Square Test; OR: Odds Ratio; CI: Confidence Interval; S: Significant at $P > 0.05$; S: Significant at $P \leq 0.05$.

Synergistic Effect of Multiple Risk Alleles

The synergistic effect of multiple risk alleles in association with T1DM (0 or 1 risk alleles versus 2, 3 or 4 alleles) is shown in table (7). Three risk alleles were significantly more risk of T1DM than 0 or 1 risk allele ($P = 0.001$).

Discussion

The table (3-2) displays the findings of the correlation between the CTLA4 1822 C/T gene polymorphism and T1DM illness. The statistical analysis indicates a significant difference between the patients and control group ($p > 0.05$) in terms of both codominance and recessive mode. However, the CC and T/C genotypes can be considered as risk factors ($OR = 0.29$ and 3.15 , respectively) with etiological fractions ($EF = 0.63$ and 0.49 , respectively). On the other hand, the TT genotype may act as a protective factor against T1DM disease. This association shows a significant difference in the dominance mode between the studied groups ($p = 0.001$). Specifically, the CC and T/C genotypes may be risk factors for T1DM, while the homozygous TT genotype is considered a protective (wild) genotype factor for T1DM ($PF = 0.38$). The findings of the present investigation are in full accordance with the results reported by Rochmah *et al.* (2022) [14]. The research found that the occurrence of the CTLA-4 1822 C/T genotype was 44% in persons with T1DM, whereas it was 72% in the control group. The frequency of the CTLA-4 1822 C/T genotype was considerably higher in the control group (odds ratio = 3.27 , 95% confidence range = 1.00 – 10.62). Nevertheless, the frequency of the CTLA-4 1822 C/T polymorphism in the T1DM patients was 44%, significantly lower than that of the control group ($OR = 3.27$, 95% CI: 1.00 – 10.62). The frequency of the CTLA-4 1822 C/T genotype was greater in the control group, indicating that this genotype may operate as a safeguard against T1DM. The table (3-4) displays the results of the association between the MicRNA21 (*rs1292037*) SNP gene polymorphism and the frequencies of genotypes and alleles in patients with T1DM and healthy control subjects. In the co-dominant mode, there was a significant difference in the distribution of genotypes between the patient and control groups ($p = 0.001$). Risk analysis indicated that the CC genotype and T/C genotype may be considered as risk factors for T1DM, with odds ratios of 0 and 0.8, respectively. The etiological fractions for

these genotypes were 0.708 and 0.83, respectively. Regarding the dominant mode analysis, the risk analysis showed that individuals with the homozygous TT genotype may have a lower chance of developing T1DM ($PF = 0.43$), making it a potentially protective genotype. The study conducted by Bayat *et al.* (2021) confirmed the findings of our current study, which revealed a higher prevalence of the heterozygous T/C genotype among T1DM patients compared to the control group ($OR = 2.74$ (1.78 – 4.27), $P < 0.0001$). Additionally, the occurrence of the C allele was also higher in patients than in the control group ($OR = 1.36$ (1.03 – 0.8), $P = 0.02$). The expression of the miR-21 gene exhibited a more than two-fold increase in patients compared to the control group ($p < 0.0001$). In the people being studied, miR-21 exhibited a significant increase in expression in those with the T/C genotype. The study findings also confirmed the conclusions of Mostahfezian *et al.*, [15]. These results align with the findings of an Iranian investigation that revealed miR-21 to be one of the most highly expressed miRNAs in endothelial cells and the pancreas in response to increased blood sugar levels. The study conducted by Ibrahim *et al.* [16], revealed a higher level of exosomal miR21 expression in the blood serum of pediatric patients with T1DM compared to the control group. In separate research conducted by Dwan *et al.* [17], it was found that there was an increase in the expression of miR-21 in a group of T1DM patients from Iraq. However, no significant difference was seen in the expression of miR-21 between T1DM patients and the control group in a sample from Sweden. This suggests that miR-21 is a component of a miRNA profile that is distinct to a certain ethnicity and is associated with Type 1 Diabetes Mellitus (T1DM). The variable expression patterns of miR-21, specifically related to insulin secretion and resistance, and its association with T1DM, have been extensively studied and acknowledged as crucial characteristics of miR-21. More precisely, the overexpression of miR-21 hinders the formation of β -cells in T1DM. Furthermore, the expression of miR-21 regulates the levels of caspases. When miR-21 is overexpressed, it leads to an increase in caspase-3 levels, which ultimately results in a decrease in cell count and cell viability. In addition, elevated levels of miR-21 lead to an augmentation in β -cell apoptosis throughout the progression of Type 1 Diabetes Mellitus (T1DM), since it selectively inhibits the translation of the bcl-2 gene Margaritis *et al.*, [18]. At the allele and genotype level in (table 3-5),

CTLA-4, T allele non significantly associated with positive family history group compared to C allele in negative family history group and with risk factor was 0.9 when compare with C allele. This result agreed with Results of (Rochmah *et al.*, 2022) (14), and Jaya *et al.*, [19], In Indonesia. At the allele and genotype level in (table 3-6), the miR-21, T allele non-significantly associated with family history positive when compared to C allele in family history negative and with

risk factor was 0.95. this result slightly agreed with result of Bayat *et al.*, [13] In Iran, and with Ganepola *et al.*, [20], In United states.

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